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Antibiotic Inhibitors of the Bacterial Ribosome

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INTRODUCTION

Many antibiotics have been identified as inhibitors of protein synthesis, acting at some step subsequent to amino acid activation. For a number of these antibiotics, it has been possible to establish the ribosome as the site of action. Studies on individual antibiotics, however, have revealed a complex diversity of inhibitory effects as well as numerous striking similarities. We would like to attempt to systematize some of the known effects of these ribosome inhibitors and to discuss certain relevant models which have been proposed for their mode of action.

Three useful references containing extensive data on antibiotics have been published. Chemical structures and physical properties of most of the antibiotics referred to below have been compiled by Umezawa and co-workers (208, 209). Information on the microbiological and biochemical aspects of antibiotic action has been compiled by Gottlieb and Shaw (76) and by Korzybski et al. (95). In addition, a large series of antibiotics has been surveyed and tested by Vazquez (214–218), Vazquez and Monro (219), and Monro and Vazquez (133) with respect to the effects on various ribosomal functions. The role of aminoglycosides in inhibiting ribosomal function and in misreading has also recently been reviewed by L. Gorini and J. Davies (70*a*).

The genetic loci for various antibiotics are indicated as follows: *str*, streptomycin; *spc*, spectinomycin; *par*, paromomycin; *nea*, neamine; *kan*, kanamycin; *neb*, nebramycin; *nek*, neomycin-kanamycin coresistance; *neo*, neomycin; *ery*, erythromycin; *ole*, oleandomycin; (*see* also Table 4). The various genotypes are designated as follows: str^s, streptomycin sensitivity; str^r, streptomycin resistance; str^d, streptomycin dependence; str^{Rd}, revertants of streptomycin dependence (double mutants).

RIBOSOMAL FUNCTION

The functional role of the ribosome in protein synthesis was recently reviewed by Watson (223). A brief summary of some reactions which play a role in the elongation of the peptide chain is presented below. These reactions were characterized in cell-free extracts prepared from *Escherichia coli*. However, the results appear to be generally valid for other bacterial systems.

Biochemical Aspects

The ribosome appears to function as a scaffold upon which the various elements involved in peptide chain initiation, elongation, and termination are brought into specific relation to each other. These steps are probably accomplished by the allocation of specific sites for the binding of messenger ribonucleic acid (mRNA), aminoacyl transfer ribonucleic acid (tRNA), and peptidyl tRNA. The factors involved in chain initiation (1) and in termination and release (17, 170*a*) have been described, and details pertaining to the function of the ribosome in relation to some of these factors have been reviewed (170).

Since available data indicate that the elongation of the peptide chain is most commonly inhibited, some of the steps in this series of reactions are summarized below. Step 1 involves activation of the amino acid and formation of aminoacyl tRNA. Step 2 involves interaction of aminoacyl tRNA with guanosine triphosphate (GTP) and "T factor" to form a T-factor-GTP-aminoacyl tRNA complex (69, 70, 156, 157). Step 3 involves binding of the T-factor-GTP-aminoacyl tRNA complex to the ribosome and localization of the aminoacyl tRNA at the "acceptor" site. Step 4 involves polymerization of a single amino acid onto the growing peptide chain by a reaction in which the peptide chain is transferred from the peptidyl tRNA bound at the peptidyl "donor" site to the incoming amino acid, bound to tRNA at the aminoacyl (acceptor site). The tRNA to which the incoming amino acid is bound thus becomes the new peptidyl tRNA. Step 5 involves translocation of the newly-formed peptidyl tRNA to the peptidyl (donor) site. Step 6 is a repetition of steps 3, 4, 5, etc., with the next incoming T-factor-GTP-aminoacyl tRNA. Steps 3, 4, and 5 have been reviewed and discussed in detail by Bretscher (12) in terms of various models, including a "hybrid-site" model which he proposes.

The roles of the two complementary soluble factors required for peptide bond formation were studied. These factors, called "T" and "G" in the studies by Nishizuka and Lipmann (142) correspond to "F. I" and "F. II," respectively, as studied by Ravel (156, 157). The T-factor from *Pseudomonas fluorescens* was further resolved by Lucas-Lenard and Lipmann (117) into a stable and unstable component referred to as "T_s" and "T_u." In *E. coli*, each is capable of complementing the G-factor with respect to peptide bond formation, but maximal activity is observed only in the presence of both T_u and T_s (50). The nomenclature used in relation to the corresponding components of F. I is "F. I_A" and "F. I_B," respectively.

The stoichiometry of GTP utilization in peptide bond synthesis remains to be further clarified. The work of Nishizuka and Lipmann (142) indicates a maximal utilization of 1 GTP [→ guanosine diphosphate (GDP) + orthophosphate (P_i)] per peptide bond. On the other hand, the

work of Ravel et al. (156, 157) and Gordon (69, 70) implies the requirement of at least 1 GTP for the formation of the T-factor-GTP-aminoacyl tRNA complex, and that additional GTP may be required for G-factor function.

There are no positive data interrelating G-factor-dependent breakdown of GTP and the GTP bound to aminoacyl tRNA, or of the exact role of the ribosome as cofactor in the G-factor-dependent breakdown of GTP. The reaction is not supported by isolated 30*S* or 50*S* subunits (J. Gordon, *personal communication*) and appears to require intact 70*S* ribosomes. It has been conjectured that the guanosine triphosphatase activity represents an uncoupled form of a reaction which normally takes place during protein synthesis (26) and that it is related in vivo to the function of peptidyl tRNA translocation. Enzymatic factors involved in the initiation of protein synthesis, referred to as F_1 and F_2 were studied by Salas et al. (166). These factors were also studied by Ohta and Thach (144), who also reviewed the properties of these factors as reported from other laboratories. It appears that these factors are utilized in the ribosomal binding of formyl methionyl tRNA but not of aminoacyl (e.g., valyl) tRNA (144). T-factor and GTP, on the other hand, are required for aminoacyl tRNA binding.

Methods of Subunit Localization of Antibiotic Action

The fact that bacterial ribosomes consist of two separable subunits with distinctly different functions provides a convenient method of subdividing the antibiotics into major classes. A number of independent criteria have been useful in relating the inhibitory function of a given antibiotic to a particular ribosomal subunit. These criteria include the following.

Reconstitution of functional ribosomes with subunits (or elements) derived from antibiotic-sensitive and antibiotic-resistant cells. Ribosomes active in protein synthesis can be obtained by reassociating complementary subunits derived from sensitive and resistant strains (30, 34). This approach can be pursued further by dissociating individual subunits into RNA and protein constituents, followed by reassociation of complementary fractions obtained from the resistant and sensitive strains. The test organisms used may be resistant and sensitive mutants of the same strain, or in some instances even of diverse bacterial species (143*a*, 204*a*).

In utilizing this approach, it is important to be able to test reciprocal combinations in order to rule out the possibility that the sensitivity or resistance observed in the hybrid combinations is merely a result of the process of dissociation and reassociation.

Binding of (labeled) antibiotic exclusively to one of the subunits. The site of fixation of an antibiotic provides presumptive evidence for the site of action, although it cannot be ruled out, a priori, that an antibiotic may bind to one subunit and act on the other. Three patterns of binding are possible.

The first pattern is binding to both subunits. The binding of tetracycline to both the 30*S* and 50*S* subunit has been reported (42, 43). According to Maxwell (126), up to 300 tetracycline molecules per 70*S* ribosome can be bound, most of it removable by dialysis. Irreversible binding, amounting to less than one molecule per 30*S* or 50*S* subunit, was also observed, and the 30*S* subunit was found to have a higher specific binding activity than the 50*S* subunit.

The second pattern is exclusive binding to one of the ribosomal subunits. Kaji and Tanaka (91) studied the binding of ^{3}H-dihydrostreptomycin to *E. coli* ribosomes. At 24 C, an average of one molecule was bound per 30*S* subunit; at 37 C, two molecules were bound. There was no detectable binding to the 50*S* subunit under the same conditions. In addition, no binding of dihydrostreptomycin to ribosomes obtained from a streptomycin-resistant strain was observed.

Exclusive binding to the 50*S* subunit has been reported for chloramphenicol (214), erythromycin (123, 201), and lincomycin (21; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). In studies on the extent of binding as a function of antibiotic concentration, a plateau level of binding over a 10-fold concentration range was observed for erythromycin and for lincomycin (F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968), corresponding to an average of one molecule per ribosome. At higher input levels, further binding was found.

The third pattern is binding to neither subunit. The binding of ^{14}C-lincomycin to *E. coli* ribosomes could not be demonstrated with the conditions (membrane filtration technique) used in studies of binding to *Bacillus stearothermophilus* ribosomes (21; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). Since lincomycin can inhibit cell-free protein synthesis in *E. coli* at concentrations 10 to 100 times those required for a comparable level of inhibition in *B. stearothermophilus*, more sensitive techniques, based on equilibrium dialysis or the use of labeled antibiotics of higher specific activity, may be useful in characterizing some properties of weaker antibiotic-binding reactions.

Competition with ribosomal binding of an anti-

biotic of known subunit specificity. This approach is useful especially if antibiotic is not available in labeled form and resistant mutants are difficult to obtain. The study of a large series of antibiotics by Vazquez (214–218) provided a set of consistent positive and negative data. Most known inhibitors of the 50*S* subunit effectively competed with ^{14}C-chloramphenicol for ribosomal binding, whereas known inhibitors of the 30*S* subunit had no effect on this binding reaction.

Demonstration of cross-resistance with antibiotics of known subunit specificity. The presence or absence of cross-resistance to antibiotics of known subunit specificity cannot be interpreted unambiguously in assigning a site of action. Thus, in *Staphylococcus aureus*, resistance to erythromycin does not necessarily confer resistance to another macrolide, spiramycin (18, 19). Similarly, cross-resistance between various amino glycoside antibiotics is also not obligatory. On the other hand, a strain of *B. stearothermophilus* selected for erythromycin resistance was found to be cross-resistant to chloramphenicol (F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). Multiple resistance to unrelated antibiotics may also result from altered membrane permeability or from resistance transfer factors.

Inhibition of a discrete function associated with an isolated subunit. Isolated 30*S* subunits can bind messenger RNA (146) and aminoacyl tRNA (92, 125). This latter step can be inhibited by tetracycline (92).

Since puromycin-dependent release involves the formation of a peptide bond between puromycin and the nascent peptide chain, it might be expected that this reaction would be inhibited by certain inhibitors of protein synthesis. Indeed, Traut and Monro (205) observed that chloramphenicol inhibits the formation of peptidyl puromycin when either 70*S* ribosomes or 50*S* subunits charged with polyphenylalanyl tRNA were used in the assay system. Isolated 50*S* subunits can also catalyze the formation of peptide bonds with formyl methionyl tRNA and puromycin, the product formed being formyl methionyl puromycin (131). This reaction is also inhibited by chloramphenicol (133).

Inhibitory effect (or lack thereof) on puromycin-dependent release of nascent peptide chains. In studies employing a peptidyl puromycin-synthesizing system, Cundliffe and McQuillen (31) observed that chlortetracyclines did not significantly inhibit puromycin-dependent release of nascent peptide chains, whereas certain inhibitors of the 50*S* subunit [e.g., erythromycin, postulated to inhibit translocation, or chloramphenicol, postulated to inhibit the actual peptide bond forming step(s)] were found to inhibit this reaction. Sparsomycin, the subunit specificity of which is as yet undemonstrated but which has been postulated to inhibit directly the formation of the peptide bond-forming step, was also found to inhibit the puromycin reaction. These observations may be understood in terms of the model proposed by Cundliffe and McQuillen (31).

According to this model, inhibitors of the 30*S* subunit can interfere with the function of the aminoacyl tRNA at the acceptor site, with the consequence that puromycin would have access to the peptide bond-forming site on the 50*S* subunit and could thereby interact with the nascent peptide chain. Inhibitors of translocation would maintain the peptidyl tRNA in the aminoacyl site, which in turn would render the peptidyl tRNA unreactive to puromycin. In addition, it would occlude the aminoacyl site and prevent access to the site from which puromycin interacts with the nascent peptide chain. Thus, inhibition of the puromycin reaction by an antibiotic implies that the antibiotic may act by inhibiting peptide bond formation, some other function of the 50*S* subunit, or both, whereas lack of an inhibitory effect suggests that the antibiotic may act only on the 30*S* subunit. Verification of the possible use of this type of assay for subunit localization studies will have to depend on further tests utilizing other classes of known 30*S* inhibitors. For a summary of the subunit specificities of the antibiotics discussed below, *see* Addendum in Proof.

INHIBITORS OF THE 30*S* SUBUNIT

At least two functions in protein synthesis can be assigned to the 30*S* ribosome subunit. (i) It provides a site of attachment for messenger RNA (143, 146, 162, 192); it is presumed that the messenger and the 30*S* subunit are displaced relative to each other during translation. (ii) It provides the site for the binding of *N*-formylmethionyl-tRNA and subsequent types of aminoacyl-tRNA before actual peptide bond formation (143); this site is related to the so-called "acceptor" or "aminoacyl" site. There is no evidence that the 30*S* subunit is in any way directly involved in peptide bond synthesis.

On the basis of these roles, one would predict that inhibition of protein synthesis on the 30*S* subunit could occur by the following mechanisms: (i) prevention of attachment of mRNA; (ii) interference with the movement of mRNA relative to the 30*S* subunit (translocation); or (iii) blockage of the acceptor (aminoacyl) site.

Subunit Localization Studies

The aminoglycosides (41, 70*a*, 87, 93) and the tetracyclines (103) are two chemically distinct classes of antibiotics; the site of action of both of these classes of antibiotics is the 30*S* subunit.

Streptomycin. Studies on the mechanism of action of streptomycin have provided a model for localization of the site of antibiotic action. The various stages in the localization of streptomycin action are listed below.

(i) By reassociation of ribosomes and soluble enzymes from sensitive and resistant strains in cell-free protein synthesizing systems, it could be shown that sensitivity or resistance was a property of the ribosomes and not of the supernatant factors (53, 182).

(ii) Reassociation of 30*S* and 50*S* subunits from sensitive and resistant, or sensitive and dependent, strains of *E. coli* yielded ribosomes which were sensitive, resistant, or dependent, depending on the source of the 30*S* subunit (30, 32, 115).

(iii) Reassociation of the 30*S* subunit from "core particles" (16*S* RNA plus 15 proteins) and "split proteins" (6 proteins) yielded 30*S* subunits which were sensitive or resistant, depending on the source of the core particle (186, 203).

(iv) Sensitive or resistant 30*S* subunits could be completely reassembled from 16*S* RNA and the 21 soluble proteins obtained from, respectively, sensitive or resistant strains (204*a*). As it is now possible to separate all 21 proteins from the 30*S* subunit, this highly specific reconstitution system has allowed the identification of the protein which confers the property of streptomycin sensitivity, resistance, or dependence on the 30*S* subunits (M. Nomura, *personal communication*; C. K. Kurland, *Personal communication*).

Streptomycin inhibits aminoacyl-tRNA binding to 70*S* ribosomes and to isolated 30*S* subunits (90, 150–153). Attempts to show specific binding of streptomycin to 70*S* ribosomes or to 30*S* subunits with a simple stoichiometric relationship have, until recently, been unsuccessful. Kaji and Tanaka (91) recently showed that ^{3}H-dihydrostreptomycin binds to the 30*S* and not to the 50*S* subunit; this binding was substantially dependent on the presence of uridine- or cytidine-containing polynucleotides. At 24 C, an average of one molecule of drug was bound to each 30*S* subunit, whereas at 37 C an average of two molecules was bound. In the presence of adenine (A)- and guanine(G)-containing polynucleotides, only one-sixth as much streptomycin was bound; this may be related to the fact that the misreading effects of streptomycin on polypeptide synthesis directed by A- and G-containing polynucleotides are not large (40). Binding of radioactive dihydrostreptomycin to 30*S* subunits obtained from a *str*^{r} strain could not be demonstrated.

Subsequent experiments by Tanaka and Kaji (200) showed that complete 30*S* subunits (23*S* core particle and split proteins) are required for the binding of dihydrostreptomycin, and that the determining factor is a protein (or proteins) of the core particle. Whereas the RNA of the core particles does not specify the dihydrostreptomycin-binding capacity of the 30*S* subunit, as might be concluded from the experiments of Traub and Nomura (204), the reconstitution experiments mentioned above (iv) show that only one protein is involved.

Tetracyclines

Evidence in favor of the 30*S* subunit as the site of action of the tetracyclines comes from the following observations. (i) Tetracyclines inhibit the binding of aminoacyl-tRNA to isolated 30*S* subunits (190) but have no effect on the puromycin-dependent release of peptides in intact cells (31). (ii) When radioactively labeled tetracycline is allowed to attach to ribosomes, the drug associates with both 30*S* and 50*S* subunits. However, the amount of tetracycline bound by the 50*S* subunits is half that bound by the 30*S* subunits (126).

The fact that tetracyclines do not inhibit the binding of ^{14}C-chloramphenicol (215) or ^{14}C-lincomycin to ribosomes (21; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968) is consistent with this evidence.

Biochemical Effects and Mechanisms of Action

A consideration of the known genetic and biochemical data on the action of aminoglycoside antibiotics, particularly streptomycin, allows us to outline plausible mechanisms for the known phenotypes. These data (mostly obtained from cell-free systems) convincingly support the notion that the ribosome is the primary site of action of these drugs, and we will interpret all results in terms of the effects of the drugs on protein synthesis on the ribosome (87).

Sensitivity. Experiments with whole cells have not added much to our understanding of the mode of action of streptomycin, and the many lesions have not been clearly interpreted in terms of the effects observed with cell-free extracts.

When sensitive bacterial strains are exposed to low concentrations of aminoglycoside antibiotics, the following effects are observed (93); (i)

cessation of respiration; (ii) excretion of nucleotides, amino acids, and potassium; (iii) inhibition of protein synthesis; and (iv) stimulation of RNA synthesis.

Since genetic evidence strongly supports a ribosomal site of action, the other effects are considered to be secondary, although the relationship between them is obscure.

Since inhibition of protein synthesis by lethal concentrations of streptomycin is observed in whole cells (net stimulation of overall peptide bond synthesis by aminoglycoside antibiotics is observed with most synthetic polynucleotides in vitro), we can examine the behavior, in cell-free extracts, of ribosomes extracted from "killed" cells. Such ribosomes respond to the synthetic messenger polyuridylic acid (poly U) and misread in the same way as do cell-free extracts from sensitive strains in the presence of added streptomycin. By contrast, ribosomes isolated from "killed" cells are inhibited with respect to the translation of natural mRNA, such as f2 phage RNA (M. Bissell and J. Davies, *unpublished data*). This effect on the translation of natural messengers may represent the state existing in streptomycin-treated cells; as is shown later, there are clear differences between the effects of streptomycin on the translation of synthetic RNA molecules and on the translation of natural RNA molecules. Such results suggest that inhibition of protein synthesis in cells results from an interaction between the ribosomal-bound drug and natural mRNA; this interaction does not occur with poly U and other synthetic polynucleotides. What function peculiar to natural mRNA is involved in the inhibitory effect of streptomycin and other aminoglycosides?

Herzog (82) found that cells treated with streptomycin contain an abnormally high content of "stuck" 70*S* ribosomes (70*S* ribosomes to which polypeptide chains are apparently attached and which are resistant to dissociation in 10^{-4} M Mg^{++}). This finding might be consistent with either of the two possibilities: (i) that streptomycin interferes with termination or release signals on natural messenger, or (ii) that streptomycin interferes with initiation and "freezes" the ribosomes as 70*S* particles.

In either case, such an inhibition of protein synthesis, combined with the fact that bound streptomycin cannot be removed easily by washing, offers a description of the lethal action of streptomycin. Gross misreading of the genetic code, if it occurs in cells, would not then be the lethal event, but may well be responsible for other lesions and for phenotypic suppression by the drug.

Effects on the rate of peptide bond formation or on polypeptide chain initiation were discussed by Likover and Kurland (114) as explanations for the inhibitory or stimulatory effects of streptomycin on bacteria, whereas Luzzatto et al. (118) presented evidence in support of a specific effect of streptomycin on initiation of protein synthesis. These authors followed a line of argument similar to that presented above, concerning the dissimilarity of streptomycin action on natural and synthetic messenger-directed syntheses. Luzzatto et al. showed that streptomycin reduces the R17 RNA-directed binding of F-methionyl (met)-tRNA and alanyl (ala)-tRNA to ribosomes, and they envisaged the formation of abortive initiation complexes (mRNA; 30*S* and 50*S* subunits) in the presence of the drug.

Complete or partial inhibition of protein synthesis by chloramphenicol or tetracycline (3, 154, 229), spectinomycin (36), amino acid starvation (H. Roth and B. D. Davis, *personal communication*), and actinomycin (C. Kirschmann and B. D. Davis, *personal communication*) can prevent or alleviate the killing action of streptomycin. This implies that the normal ribosomal cycle in protein synthesis (122) must be in operation for the aminoglycosides to exert their bactericidal effect. Since puromycin inhibition of protein synthesis requires a continued ribosome cycle, it is not surprising that puromycin inhibition of protein synthesis does not protect against the bactericidal effect of streptomycin (224, 229).

Any theory of aminoglycoside action must take into account the dominance of streptomycin sensitivity over resistance (106); two explanations have thus far been offered. The first is based on the notion that gross misreading of the genetic code is responsible for killing (38). The second and, at this moment, more likely proposal (105) suggests that streptomycin interferes with initiation or termination; only one sensitive ribosome on each polysome would then be necessary to inhibit the translation process in the presence of streptomycin.

Another characteristic property of aminoglycoside drugs is phenotypic suppression (71). This property is not confined to resistant cells; in the presence of sublethal concentrations of aminoglycosides, sensitive cells which carry nonsense (73, 225) or missense (225; J. Davies, *unpublished data*) mutations can be suppressed to low levels. This effect can be detected easily by spreading bacteria on media lacking the required growth factor and placing antibiotic sensitivity discs or crystals of the drug on the media surface "Halos" (implying slow growth)

around the discs or crystals suggest phenotypic suppression of the particular mutation. Nonsense and missense (148, 159) mutations in bacteriophage can also be phenotypically suppressed; recently, it has been shown that these drugs can enhance the efficiency of genetic suppressors of amber (L. Gorini, *personal communication*) and missense (J. Davies, *unpublished data*) mutations.

Resistance. Experiments with cell-free polypeptide synthesis suggest that there is one site on a sensitive ribosome to which streptomycin binds in order to derange protein synthesis; any mutation which eliminates this site, or alters it in such a way that the bound drug can no longer exert its effect, would constitute a mutation to resistance. The production of mutants resistant to a high concentration (1 mg/ml) of streptomycin by single-step mutation is also consistent with a single site of action, although Kaji and Tanaka (91) found that one or two molecules of drug can bind to sensitive ribosomes, depending on the temperature of the incubation. Kaji and Tanaka also showed that the drug-binding site was eliminated in the *str*^{r} strain that they used. This finding that mutation to streptomycin resistance eliminates the active site might be considered inconsistent with the fact that *str*^{r} cells are often phenotypically suppressible by the drug and must still possess a ribosomal binding site for it. It is possible that both types of mutation to resistance exist.

Apart from their behavior in cell-free protein synthesizing systems, the only other currently known distinguishing feature between *str*^{s} and *str*^{r} ribosomes is described by Leon and Brock (108). Centrifugation of 70*S* ribosomes at high temperatures (58 to 64 C) causes dissociation of these particles into subunits, with the destruction of the 30*S* subunit (presumably by nuclease action); Leon and Brock examined the effects of streptomycin or neomycin on the dissociation of sensitive and resistant ribosomes at 61 C. Both streptomycin and neomycin prevented breakdown of *str*^{s} ribosomes under these conditions, whereas only neomycin protected ribosomes from the *str*^{r} strain. In addition, low concentrations of streptomycin or neomycin were capable of maintaining a significant proportion of the ribosomes from a sensitive strain as intact 70*S* particles in 10^{-3} M Mg^{++}; neomycin, but not streptomycin, protected ribosomes from a *atr*^{r} strain at this suboptimal magnesium concentration.

Surprisingly, dihydrostreptomycin, which is indistinguishable from streptomycin in its antibacterial effects, was less effective in its ability to prevent thermal dissociation of ribosomes; this suggests that a nonspecific effect of the drug, unrelated to its mode of action, may be the source of these findings.

Wolfe and Hahn (226) extended the studies of Leon and Brock; they showed that ribosomes prepared from a sensitive strain which had been exposed to streptomycin were more heat-stable at 52 C than were ribosomes obtained from untreated control cultures. The melting profile of ribosomes from a resistant strain, although slightly different from that of the sensitive parent, was only slightly altered by growth in the presence of the drug. It would be of interest to learn whether these "stabilized" ribosomes bear any relationship to the abortive initiation complexes of Luzzatto et al. (118).

Dependence. Bacterial mutants with an absolute requirement for an aminoglycoside antibiotic, such as streptomycin, paromomycin, neamine, and the neomycins (67, 180; W. Szybalski and J. Cocito-Vandermeulen, Bacteriol. Proc., p. 37–38, 1958), are found among the resistant mutants when a sensitive strain is plated on media containing antibiotic. When grown in the absence of the drug (184), such mutants show a typical pattern of cessation of macromolecule synthesis, characterized by a decrease in protein synthetic activity but undiminished deoxyribonucleic acid (DNA) and RNA synthesis; studies on the deprival of drug-dependent strains first led Spotts and Stanier (185) to propose a ribosomal site of action for the aminoglycoside antibiotics.

Two questions are of importance with respect to drug dependence, neither of which has been satisfactorily answered. First, it would be interesting to know whether mutations to dependence are related to mutations to resistance. On the basis of genetic data obtained mainly with *E. coli* (81, 139), it is thought that these characteristics are allelic, and it has been suggested that *str*^{d} mutants constitute a class of *str*^{r} mutants.

Consistent with this conclusion is the finding by Luzzatto et al. (119) that transduction of a *str*^{r} allele from one strain of *E. coli* to another resulted in a *str*^{d} phenotype. These authors propose the existence of a "modifier" locus which interacts with a streptomycin allele to express the phenotype peculiar to this interaction in the strain in question. Whether this is a general phenomenon must await further studies. However, although *str*^{r} strains show no cross-resistance with other aminoglycosides, cross-dependence can be clearly demonstrated. Thus, *str*^{r} strains are not resistant to paromomycin or neamine, whereas *str*^{d} strains can grow on these drugs (74; W. Szybalski and J. Cocito-Vandermeulen, Bacteriol. Proc., p. 37–38, 1958). Secondly, in view of the ease with which other

aminoglycosides can substitute for streptomycin for the growth of dependent strains, the phenomenon of dependence can be explained either by the use of aminoglycosides as cationic structural elements required for ribosome assembly or subunit association, or by the more subtle but direct use of the drug in one of the various biochemical reactions which take place on the 30S subunit. Certain experiments suggest that low concentrations of streptomycin (10^{-5} M) can maintain the ribosomes of a streptomycin-dependent strain as 70S particles in 10^{-3} M Mg^{++}, and that this apparent reassociation results in protein synthetic activity in vitro, when poly U or bacteriophage MS2 RNA is used (J. Davies, Proc. Intern. Congr. Biochem., 6th Abstr. I-40, 1964). The streptomycin requirement for maximal rate of protein synthesis on ribosomes of dependent strains was most clearly demonstrated in the experiments of Likover and Kurland (115). By use of incubation mixtures in which the divalent cation content was made up of both calcium and magnesium, Likover and Kurland were able to demonstrate that optimal polypeptide synthesis on *str*^{d} ribosomes was substantially dependent on the addition of low concentrations of streptomycin; this requirement was shown to be related to the 30S subunit of *str*^{d} strains and could not be satisfied by a related aminoglycoside, such as neomycin.

If misreading is a necessary requirement for the growth of *str*^{d} strains, one might expect that other agents which induce misreading would satisfy the requirement. Support for this notion comes from the finding that *str*^{d} strains grow on neamine (W. Szybalski and J. Cocito-Vandermuelen, Bacteriol. Proc., p. 37–38, 1958) paromomycin, or 3% ethyl alcohol (59); such results prompted Gorini et al. (74) to consider dependence as a general effect and that *str*^{d} mutants should be redefined as drug-dependent. Since all of these agents induce translation errors in vitro, it might be reasoned that a certain level of induced ambiguity is necessary for the growth of drug-dependent strains; 5-fluorouracil, which is also thought to produce altered proteins (15), cannot satisfy this requirement (J. Davies, *unpublished data*). The binding of radioactive streptomycin or dihydrostreptomycin to *str*^{d} ribosomes has been examined but has not provided any conclusive results (227).

Drug-independent revertants from drug-dependent strains. If a *str*^{d} mutant is plated on media lacking the required drug, colonies of drug-independent revertants appear. These strains have a wide range of phenotypes, from sensitivity to high-level resistance, and can be shown in *E. coli* to be produced by a second mutation by back-crossing (81). Brownstein and Lewandowski found that one such streptomycin-sensitive revertant strain behaves exactly like a *str*^{s} strain in vitro, as measured by inhibition of polypeptide synthesis and patterns of ambiguity (14). In addition, this revertant accumulates incomplete 50S ribosomal particles concomitantly with the normal synthesis of ribosomes during exponential growth (109; B. Brownstein, *personal communication*). It would thus appear that, in this revertant, mutation from drug dependence to drug independence involves a change in the 50S and not the 30S subunit, despite the fact that the original mutation to dependence occurred in the 30S subunit.

We will now present a discussion of the salient properties of certain antibiotics affecting the 30S subunit.

Streptomycin. The addition of streptomycin to an in vitro polypeptide synthesizing system with *str*^{s} extract can produce the following effects.

(i) It can inhibit polypeptide synthesis directed by various types of natural mRNA at Mg^{++} concentrations optimal for peptide bond formation (49, 169, 221).

(ii) It can inhibit the incorporation of certain synthetic polymer-directed incorporations; e.g., poly U-phenylalanine (52, 182), poly (AC)-histidine and threonine, and poly (AG)-arginine and glutamic acid (40).

(iii) It can stimulate polypeptide synthesis directed by various types of natural messenger RNA at supra-optimal magnesium concentrations (221).

(iv) It can stimulate the incorporation of amino acids not coded for by certain synthetic polymers; e.g., poly U-isoleucine, serine; poly (C-histidine, threonine, and serine (37, 38). The net result with synthetic polymers is that streptomycin produces a substantial stimulation of peptide bond synthesis. This gives rise to an anomalous situation in which poly U-directed phenylalanine incorporation is inhibited by streptomycin (52, 182), but overall peptide bond synthesis is increased when isoleucine, serine, and tyrosine are present in the incorporation system. Exceptions are the incorporations directed by poly (AC) and poly (AG); in these two cases, streptomycin inhibits overall polypeptide synthesis without inducing misreading (40)

(v) It can promote the activity of denatured DNA, ribosomal RNA, tRNA, and other non-messenger-like polymers, such as polyinosinic acid (I) as templates for polypeptide synthesis (120, 134).

The chemical basis of these various effects is not known, and studies are complicated by the

fact that variations in reaction conditions can affect considerably the nature of the results. This is obvious in the cases of (i) and (iii) where alterations in the magnesium ion reverse the effect of the drug; changes in tRNA concentration can also influence the misreading patterns (39, 153). Perhaps the most striking example of the effect of environment comes from the recent experiments of Likover and Kurland (114), who found that streptomycin had no misreading effects in an incorporation system with highly purified ribosome and supernatant fractions. Translation errors could now be introduced in the streptomycin-containing system by the addition of small amounts of denatured DNA. As these authors pointed out, the phenotype of the ribosome is conserved (that is, sensitivity, resistance, and dependence, as measured by inhibition or stimulation of protein synthesis, were dictated by the phenotype of the parent cultures); it is not clear whether the added DNA is a true or substitute cofactor. This finding further complicates the interpretation of the in vivo effects of streptomycin in terms of the phenomena observed in cell-free extracts.

The streptomycin antibiotics inhibit polypeptide synthesis and cause misreading in sensitive cell-free systems at concentrations which are compatible with the bactericidal concentrations (36, 52). When this is calculated in terms of the number of drug molecules per ribosome for maximal effect, this ratio is close to one with streptomycin and its close derivatives and also with paromomycin.

The occurrence of both inhibition and misreading in vitro is well defined (*see above*); the two effects are probably separate and distinct expressions of drug action, although whether one, or both, or neither is responsible for the antibacterial action of the drug has not been determined. There is an apparent relationship between misreading, killing action, and phenotypic suppression in that the aminoglycoside drugs which do not cause misreading (e.g., spectinomycin) are static drugs only. They do not support the growth of str^d strains, nor do they cause phenotypic suppression. However, the fact that extensive misreading can occur in certain bacteriol mutants without causing cell death argues against gross misreading as such as the primary cause of killing (72).

At present, most of the evidence favors the notion that the lethal effect of streptomycin is the result of irreversible binding of the drug to ribosomes and the subsequent interference with some phase of protein synthesis on the ribosome. Most of the evidence in support of this theory comes from in vitro experiments, and some of the effects of streptomycin on whole cells are not clearly interpretable in these terms alone. Amino acid substitution induced by streptomycin in whole cells has not yet been demonstrated as such, and protection of streptomycin-treated cells by inhibition of protein synthesis or by a shift from aerobic to anaerobic conditions has not been explained satisfactorily.

Cohen and his group (23, 57, 187) directed attention to the ability of streptomycin to bind to DNA (the drug is a good precipitating agent for nucleic acids) and proposed that the drug exerts its lethal effect by reason of such an interaction. Stern et al. (187) studied the effect of streptomycin on starved amino acid auxotrophs in order to demonstrate that the lethal effect of streptomycin can be expressed in the absence of protein synthesis; this would obviously eliminate gross misreading as the lethal event. It is not easy to reach unambiguous conclusions from experiments involving the effects of antibiotics during conditions of amino acid starvation, since the experiments of Roth and Davis (*personal communication*) with a wide selection of amino auxotrophs showed that not all of these strains were protected against the lethal action of streptomycin when growth was prevented by amino acid starvation. Mutants with various degrees of "leakiness" would naturally give misleading results. Various degrees of protein turnover after amino-acid starvation would also complicate the interpretation of these findings.

Stern et al. (187) also suggested that, as a consequence of the binding of streptomycin to DNA, an abnormal RNA species is formed, which, in some undefined manner, has adverse effects on the cell. These interpretations do not take into account the genetic and biochemical evidence which supports a ribosomal site of action for streptomycin, and alternative explanations of streptomycin resistance or dependence in terms of the DNA model are not readily apparent. If an interaction between streptomycin and DNA is critical for the lethal action of this drug, then the reduced derivative dihydrostreptomycin would be expected to act in the same way, since the antibacterial effects of these two drugs are identical. However, dihydrostreptomycin does not bind to nucleic acids to nearly the same extent as streptomycin (135).

A resolution of this difference in opinion may be related to the fact that the translation and transcription processes are thought to be very closely interrelated (13, 162). If streptomycin (and dihydrostreptomycin) interferes with initiation of translation, the abortive complex so formed might also interfere with transcription and produce effects similar to those described by

Cohen et al. (23, 57, 187). Protein synthesis would not then be required for streptomycin killing.

Other aminoglycoside antibiotics related to streptomycin in action. The drugs listed in Table 1 have not been studied to the same extent as streptomycin because of the difficulty in obtaining resistant bacterial mutants. It has not, therefore, been possible to perform the same genetic and biochemical analysis as in the case of streptomycin. Some progress, however, has been made recently in genetic studies of mutants resistant to or dependent on paromomycin (L. Gorini, *personal communication*) and neamine (J. Davies, *unpublished data*), which map in the 30*S* region.

The drugs in Table 1 were tested for their effects on polypeptide synthesis in vitro, and, like streptomycin, they caused misreading or inhibition, or both, of peptide bond formation, depending on the nature of the mRNA and Mg^{++} concentration used. Unlike streptomycin, however, the antibiotics neomycin, kanamycin, and gentamicin produced much higher levels of misreading and did not show a simple stoichiometric drug-to-ribosome ratio for this effect (Fig. 1). The concentration-activity curves seem to be characteristic for each drug (Fig. 1) and may imply that these drugs work by interacting with more than one site on the ribosome or with more than one component of the protein-synthesizing machinery (37).

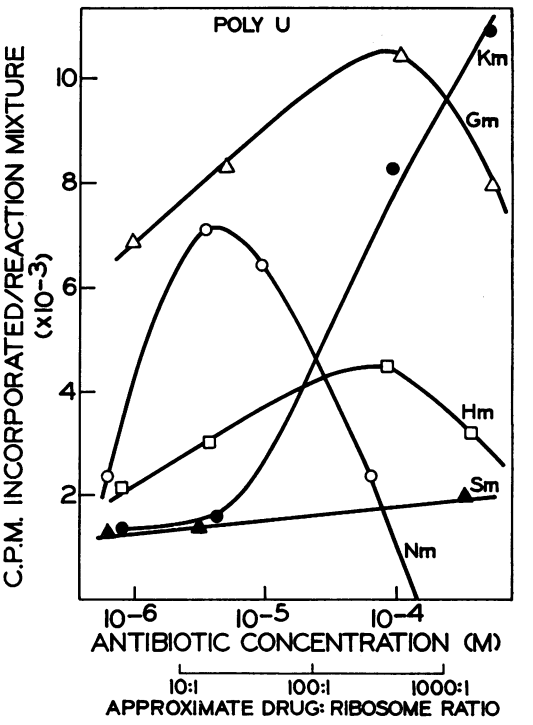

FIG. 1. *Effect of increasing drug concentrations on total misreading of poly U. For experimental conditions, see reference 37.*

TABLE 1. *Aminoglycoside antibiotics which cause translation errors*

Antibiotic	Group
Streptomycin	Streptomycin
Dihydrostreptomycin	
Bluensomycin	
Neomycin B	Neomycin
Neomycin C	
Paromomycin	
Neamine	
Paromamine[a]	
Kanamycin A	Kanamycin
Kanamycin B	
Gentamicin	
Nebramycin	
Hygromycin B	

[a] There is some disagreement over the misreading properties of paromamine. Masukawa and Tanaka (124) reported that this aminoglycoside causes translation errors, but Yamada and Davies (*unpublished data*) found that over a wide concentration range paromamine has no inhibitory or misreading effects.

When tested at sublethal concentrations against sensitive strains, these other aminoglycosides are all capable of phenotypic suppression of nonsense and missense mutations (73, 225). Paromomycin and neamine appear to have a close relationship with streptomycin, as mutants selected as dependent on one of these drugs display cross-dependence on the other two. However, str^r, pm^r, and nea^r strains of *E. coli* are not generally cross-resistant (J. Davies, *unpublished data*).

Spectinomycin. Spectinomycin is an aminoglycoside which contains the sugar residue actinamine (84), an isomer of streptamine. (Deoxystreptamine or streptamine residues are characteristic of the aminoglycosides capable of misreading.) Spectinomycin was studied during the course of a general screening of aminoglycoside drugs (36); it was found to differ from those aforementioned to the extent that it does not kill cells and its inhibitory effects, in contrast to those of streptomycin, can be reversed by washing the affected cells. Although an effective inhibitor of protein synthesis in cells and in extracts, spectino-

mycin does not induce errors in translation. Mutants resistant to spectinomycin have been isolated, but dependence has not been found and the drug does not allow phenotypic suppression. Spectinomycin does not support the growth of *str*^{d} mutants, and there is no cross-resistance between spectinomycin and streptomycin.

Some similarities between the two drugs have been noted; mutants resistant to spectinomycin are single-step mutants to high-level resistance (1 mg/ml) and spectinomycin inhibits polypeptide synthesis maximally in vitro at a drug-to-ribosome ratio of 1 (36). Furthermore, the *spc*^{r} and *spc*^{s} phenotypes are determined by the 30*S* ribosomal subunits and map, like the *str* alleles, between the *aroE* and *aroB* loci on the *E. coli* genetic map (36, 54; P. Anderson, *personal communication*).

One of the impressive features of the action of spectinomycin in vitro is that its inhibitory effect depends strikingly on the nucleotide composition of the messenger. Incorporation directed by natural mRNA was 70 to 80% inhibited by spectinomycin (1 μg/ml), whereas poly U- and poly A-directed incorporations were quite inert, even at drug concentrations in excess of 100 μg/ml. Introduction of C, and especially G, residues into polymers containing A and U notably increased the degree of inhibition, and inhibition increased with increasing G content; some UG polymers were affected almost to the same extent as natural RNA by the drug (Table 2). This base specificity was assumed to be of considerable importance by Anderson et al. (4), who proposed that spectinomycin interacts with G-sequences or certain G-containing codons in natural messengers.

Spectinomycin is a strong inhibitor of amino acid incorporation directed by poly I. Although poly I-directed incorporation has some unusual features, such as an abnormally high magnesium ion requirement (2.7×10^{-2} M), which are not understood, the poly I system lends itself well to a study of the inhibitory effect of spectinomycin (4); the results of additional experiments carried out to define the mode of action of spectinomycin prompted the conclusion that the drug blocks some aspect of the translocation process. This conclusion was arrived at by a process of elimination, since the drug was not found to affect codon recognition, aminoacyl-tRNA binding, peptide initiation or release, or peptide bond formation (as measured by the puromycin reaction). Since the system of Cundliffe and McQuillen (31) presently provides a reasonable model in which to study translocation, it would be of interest to test spectinomycin in this way. It appears, however, that the conclusion that spectinomycin affects translocation is inconsistent with the interpretations of Cundliffe and McQuillen concerning 30*S* and 50*S* inhibitors.

TABLE 2. *Effect of spectinomycin on the activity of heteropolynucleotide messengers*

Nucleotide input ratio of messenger	[^{14}C]Amino acid[a]	Radioactivity incorporated (counts/min)		Inhibition by Spectinomycin
		No drug	Spectinomycin (1 μg/ml)	
				%
None	Phe (10 μc/μmole)	11	17	
U		4,210	4,090	3
9U:1G		2,290	1,650	18
3U:1G		1,290	880	32
1U:1G		413	237	44
None	Gly (80 μc/μmole)	31	24	
U		28	41	
9U:1G		333	227	35
3U:1G		1,600	860	47
1U:1G		3,420	1,390	60
None	Lys (10 μc/μmole)	112	105	
A		513	497	4
3A:1G		941	458	58
None	Phe (10 μc/μmole)	51	56	
U		9,850	10,600	
3U:1C		4,220	3,830	10
1U:3C		716	557	24

[a] Phe, phenylalanine; gly, glycine; lys, lysine.

Generalizing from their results leads one to the conclusion that 30S inhibitors might not be expected to affect translocation, even though spectinomycin is proposed to act in this manner. This inconsistency may be explained when we know more about the chemical nature of translocation; however, there seems to be no a priori reason why inhibitors on the 30S subunit should not affect this step.

Kasugamycin. Kasugamycin is an aminoglycoside which contains a D-inositol residue and an unusual amidine group. Like spectinomycin, it does not contain a streptamine or a deoxystreptamine residue (189). It differs from the other aminoglycosides in that the drug is more effective against gram-positive than gram-negative organisms; this may simply be a question of permeability. The effects of kasugamycin on protein synthesis in cell-free extracts have been studied in some detail (196, 198). Kasugamycin was found to reduce the binding of aminoacyl-tRNA to 70S ribosomes in the presence of polynucleotides; the corresponding experiments with 30S subunits have not been reported. It is not entirely clear that this drug is an inhibitor of the 30S subunit, but it has been included in this section because of its chemical similarity to other aminoglycosides. It should be noted that high concentrations of kasugamycin were required to demonstrate inhibition of polypeptide synthesis (640 μg/ml gave 91% inhibition). Since *E. coli* extracts were used, this may reflect the tolerance of this organism to the drug.

Tetracyclines. Like most studies with antibiotics, experiments with tetracycline on whole cells have uncovered a multiplicity of lesions, most of them mutually inconsistent (102). Although some effects of the tetracyclines might be due to their chelating properties, the finding that these drugs are effective inhibitors of polypeptide synthesis in vitro to a great extent determined the approach to studies on their mode of action (55, 60, 61, 63, 75, 83, 161, 219). The concentrations required for inhibition in vitro were low (about 10 μg/ml) and similar to the concentrations required for inhibition of bacterial cell growth; no significant differences were detected between the various tetracyclines and their derivatives. Examination of the binding reaction showed that the drug reduces the binding of *N*-acetylphenylalanyl-tRNA to a poly U-ribosome complex by about 50% (188). (Unlike chloramphenicol and spectinomycin, tetracycline is an effective inhibitor of poly U-directed synthesis; 10 μg/ml produces an inhibition of 80 to 90%.) The drug does not detectably inhibit the binding of poly U to ribosomes (103).

Further experiments of this type showed that tetracycline is an effective inhibitor of the binding of aminoacyl-tRNA to messenger RNA-30S subunit complexes (190); this suggests that tetracycline prevents the binding of tRNA to the acceptor (amino acid) site on the 30S subunit. More convincing evidence in support of such a mechanism of action for tetracycline is provided by Gottesman (75), Lucas-Lenard and Haenni (116), and Sarkar and Thach (168; S. Sarkar, Federation Proc., p. 398, 1968), who demonstrated that tetracycline can inhibit the binding of lysyl-tRNA or F-met-tRNA to the ribosomal acceptor site (the A site). F-met-tRNA binding was especially sensitive to tetracycline, a 50% inhibition of binding being produced by a drug concentration of 5×10^{-4} M. Tetracycline does not affect peptidyl transferase action on the 50S subunit (205).

Studies on the binding of radioactive tetracycline to ribosomes and other components of the protein-synthesizing machinery are not definitive, but the fact that more drug binds to the 30S than to the 50S subunit is thought to support the notion that tetracycline acts primarily on a process involving the 30S ribosome subunit These binding experiments appear to be complicated by the fact that the drug can bind strongly to mRNA and other RNA-containing constituents of the in vitro system (25, 42, 43); however, it has been clearly demonstrated that only ribosome-bound antibiotic is inhibitory (43). When ribosomes are first treated to remove bound mRNA, substantial amounts of tetracycline remain attached, but only a small fraction of this material is irreversibly bound. This irreversibly bound material amounts to less than one molecule of drug per ribosome; it is found attached to both the 30S and 50S subunits, although the specific activity of the 30S subunits is twice as large as the activity of the 50S (126).

The binding of tetracycline to ribosomes is dependent on the concentration of the magnesium and potassium ions, and it is not clear if the attachment is completely irreversible or if it is exchangeable. Since the inhibitory effects of tetracycline on whole cells can be reversed by washing, this irreversibly bound drug may not be responsible for the inhibitory effects. The experiments of Day (42, 43) also indicated an apparent "irreversible" binding of tetracycline to ribosomes, but this material appears capable of exchanging between the ribosomal subunits.

Some bacterial mutants resistant to tetracycline have been isolated (102, 160), but no convincing demonstration of a tetracycline-resistant protein-synthesizing system in vitro

has been presented. Most of the tetracycline-resistant strains isolated to date seem to be resistant by virtue of alterations in permeability or by enzymatic inactivation of the drug.

Genetic Studies

The most striking feature of the aminoglycosides, as exemplified by streptomycin, is that a bacterium can have three phenotypically distinct but genotypically related responses to these drugs: sensitivity, resistance, or dependence. (Dependence has not been found for all of the aminoglycosides.) Genetic analysis of these responses has been carried out in detail for streptomycin; it appears that sensitivity, dependence, and single-step, high-level resistance are determined by multiple alleles of a single genetic locus (81, 139), but the possibility of closely linked, multiple genetic loci cannot be excluded. By dependence, we mean "classical" dependence, when an absolute requirement for the drug is shown under normal growth conditions. This is distinct from conditional dependence, when drug is required only in place of a certain growth factor.

Phenotypic aspects. There are several mechanisms by which a drug-resistant bacterium can arise. Mutation can restrict the uptake of the drug, eliminate or alter the binding of the drug to its target site, or produce a mechanism by which the drug is inactivated. Very little, if anything, is known about bacterial mutants which have impaired transport for the aminoglycoside antibiotics, and this discussion is confined to resistance arising in two ways.

The first way resistance can arise is from an alteration of a ribosome caused by a mutation in the target site of the drug; this renders this target immune to the drug's action. As far as can be ascertained at the present time, such mutations confer high level resistance (1 mg/ml) and typical examples are the single-step, high-level resistance mutations to streptomycin and to spectinomycin. The second way resistance can arise is from mutations which cause inactivation of drugs. It is difficult to imagine how a single mutation can produce an enzyme capable of inactivating a drug, unless it occurred by alterations in the specificity of a pre-existing enzyme. Such a mechanism of resistance by inactivation, has, however, been well characterized in the case of resistance mediated by resistance-transfer factors (RTF). This transmissible form of resistance usually only confers resistance to low drug concentrations (about 25 μg/ml). RTF resistance is discussed in more detail below.

The level of resistance conferred depends on the drug being studied. Although mutants resistant to streptomycin concentrations as high as 5 to 10 mg/ml can be obtained without great difficulty, single-step "high-level" resistance to kanamycin, neamine, and other aminoglycosides is generally only to drug concentrations of 200 μg/ml or lower (about 30 μg/ml in the case of neomycin). There is reason to believe that some of the aminoglycosides are less specific in their mode of action when high drug concentrations are used (37).

Most of these resistant phenotypes can be readily demonstrated in whole cells and in cell-free extracts; the 30*S* ribosome subunit has been implicated in the case of streptomycin (30, 34), spectinomycin (36), and neomycin (J. Davies, *unpublished data*). The exact chemical alteration responsible for such resistance is not known, although it has been shown that a mutational change in a single ribosomal protein is involved in streptomycin resistance and dependence since the "streptomycin" protein has been isolated (M. Nomura, *personal communication*; E. Birge and C. G. Kurland, *personal communication*). It is possible that resistance arises by a mutation which destroys the site of binding of the drug, or by modification of ribosome structure such that the drug can no longer have any marked effect on ribosome function, even though it is present in its normal or altered site. As has been stated earlier, there is some evidence to support both possibilities.

In addition to the fact that resistant mutants are refractory to the drug in question (for cross-resistance, *see below*), there are other typical phenotypes of resistant cells. Mutation to aminoglycoside resistance frequently gives rise to pleiotropic effects which can take the following forms: (i) bacteriophage restriction and modification (29, 107); (ii) reduction or loss of the drug-dependent phenotypic suppression shown by the parent sensitive strain (5, 7, 63); (iii) modification (reduction or elimination) of the properties of a pre-existing genetic suppressor (35, 101, 105, 149; L. Gorini, *personal communication*).

It is possible to distinguish three classes of nonidentical *str*^{r} mutations relative to their effect on the efficiency of suppression by an amber suppressor gene. These classes show strong restriction, weak restriction, or no restriction of the suppressor function (149; L. Gorini, *personal communication*). Unfortunately, there has been little study on the nature of these effects, and an explanation for the magnitude of some of these effects in biochemical terms is not readily apparent.

Resistance to aminoglycoside antibiotics was

convincingly demonstrated in cell-free extracts of resistant *E. coli* cells, the drug causing no inhibition of polypeptide synthesis. Misreading at a low level was found in certain (competent for phenotypic suppression) resistant mutants but not in other (incompetent for phenotypic suppression) resistant mutants (5).

There appears to be a high degree of specificity in mutations to aminoglycoside resistance. Very little cross-resistance has been detected, except among closely related drugs. Thus, *E. coli* mutants selected for high-level resistance to streptomycin, dihydrostreptomycin, or bluensomycin are completely cross-resistant to each other and sensitive to other aminoglycosides such as neomycin and kanamycin. Spectinomycin shows no cross-resistance with any other aminoglycoside tested. Cross-dependence, however, is well known and Gorini et al. prefer to call this "generalized drug dependence."

Cross-resistance and cross-dependence have also been studied in strains of *S. aureus* (180, 181), but it is not known whether resistance is due to a ribosomal mutation in these strains. Mutants of *S. aureus* were obtained by serial passage through increasing concentrations of antibiotic and were found to display a wide variety of patterns of cross-resistance and cross-dependence with neamine, neomycin B, neomycin C, paromomycin, and streptomycin (180, 181).

Bacterial mutants which are streptomycin-dependent have an absolute requirement for streptomycin. This requirement can be satisfied by certain other aminoglycoside antibiotics (e.g., paromomycin or neamine) and also by the presence of aliphatic alcohols such as ethyl alcohol (3%) in the growth medium (59). *Str*^{d} mutants cannot be selected directly; during the screening of mutants arising after plating large numbers of sensitive colonies on streptomycin agar, a substantial number of the "resistant" colonies are, in fact, dependent. The yield of *str*^{d} mutants is found to vary from strain to strain; in certain cases, it seems that all of the mutants arising by such a selection are *str*^{d} (67). This type of analysis of mutation rate is somewhat restricted as it can score only those *str*^{d} mutants which arise in the culture medium before plating. Any *str*^{d} mutant arising on the plate would not survive because of the known dominance of sensitivity. The same would be true for resistant mutants, although these mutants would have some advantage in being capable of survival and propagation in medium free from drug. In the case of paromomycin, all of the clones arising after selection on an antibiotic plate are dependent (74), whereas neamine is like streptomycin in that both resistant and dependent classes appear (J. Davies, *unpublished data*).

As in the case of mutation to drug resistance, mutation to drug dependence also has pleiotropic effects and additional nutritional requirements are common. Mutation to streptomycin dependence also carries the property of phenotypic suppression. However, the characteristics of phenotypic suppression in dependent strains appear to be different from those encountered in resistant strains. In all of the *str*^{d} strains thus far examined, phenotypic suppression is enhanced as compared to the sensitive parent. (J. Davies, *unpublished data*). It should be recalled that mutation to resistance often restricts this property. Phenotypic suppression by dependent strains is a very generalized form of weak suppression which can affect most of the amber, ochre, and UGA mutants, as well as certain missense mutants of phage T4 and the *lac z* gene. This suppression is patently a property of the mutation to dependence, since independent revertants selected from the dependent strains do not have this property, either in the presence or in the absence of streptomycin (J. Davies, *unpublished data*). This might be explained by saying that when a sensitive ribosome undergoes a mutation which affects its response to an aminoglycoside antibiotic, it can assume either a configuration which restricts ambiguity (resistance) or a configuration which favors ambiguity in translation (dependence).

In vitro experiments have been used to demonstrate both the resistant and dependent properties of ribosomes from drug-dependent strains (53, 115; J. Davies, Proc. Intern. Congr. Biochem., 6th, Abstr. I-40, 1964). Special conditions such as the use of "starved" streptomycin-dependent strains (J. Davies, Proc. Intern. Congr. Biochem., 6th, Abstr. I-40, 1964) or incubation mixtures containing Ca^{++} (115) must be employed to demonstrate a requirement for streptomycin for in vitro polypeptide synthesis.

Drug-independent mutants can be readily selected from drug-dependent mutants by plating on media in the absence of drug (8, 56, 66, 81, 85). There is often a substantial background growth resulting from the residual streptomycin, but this can be overcome by first washing the cells well and allowing them to propagate in drug-free medium for 10 to 12 hr before plating. A more convenient selection for drug independence can be made when the drug-dependent strain developed a nutritional requirement during the original selection. This facilitates selection of drug-independent mutants, since they generally lose the nutritional requirement of the parent dependent strain and selection on suitable media

eliminates any background growth of the parental strain. The drug-independent mutants of *E. coli* also exhibit a wide range of phenotypes from drug sensitivity to high-level resistance. These strains are all double mtuants, and back crosses have shown that the original *str*^{d} mutation is still present (81). It is considered that these are allelic suppressor mutations of *str*^{d}. However, they do not have the properties of any known genetic suppressor of nonsense or missense (14; J. Davies, *unpublished data*). The mutation to drug independence might equally well be an intragenic or extragenic suppressor mutation, although good operational descriptions or testable models of these phenotypes have not been presented. True revertants of *str*^{d} strains to *str*^{s} have been reported in *Bacillus* (56), *Proteus* (85), and in a highly mutable strain of *Salmonella typhimurium* (66).

The most comprehensive genetic studies of *str*^{d}, *str*Rd, and indeed of the streptomycin locus were carried out by Hashimoto (81), by a transduction analysis of such mutations. Fine structure mapping was not possible, but he was the first to show the close genetic relationship between *str*^{r}, *str*^{d}, and *str*Rd.

Very little is known of the biochemical nature of the *str*Rd mutation, but the properties of the ribosomes are apparently distinct from those of sensitive or dependent strains (8). In vitro experiments with a phenotypically sensitive *str*^{d} *str*Rd strain showed that it behaved like a sensitive strain; however, a lesion in ribosome synthesis or assembly was also reported in the same mutant (14, 109).

Recent experiments on the physiology of *str*^{d} strains showed that during starvation for streptomycin, preferential inhibition of certain enzymes occurred (68). Synthesis of β-galactosidase and alkaline phosphatase was markedly inhibited, and catabolite repression could account for only part of this decrease. There was no evidence for the production of immunologically active cross-reacting material. (This might be expected if translational misreading occurred.) The authors suggested that, during streptomycin-deprived growth, the ribosomes of *str*^{d} strains lose the ability to translate certain messages as a result of a failure to recognize certain critical codons near the 5'-end of the mRNA. It is difficult to imagine how such a general mechanism would lead to such selective effects.

Genotypic aspects. Fine structure mapping of these regions has not yet been accomplished, because it has not been possible to devise a selection technique capable of detecting the rare sensitive recombinants from crosses between resistant and dependent mutants. The genetic markers for streptomycin phenotypes are found to map between the *aroE* and *aroB* loci on the *E. coli* linkage map; the determinants for spectinomycin resistance, paromomycin dependence, and neamine resistance map in the same region (Fig. 2). With streptomycin (30, 34) and spectinomycin (36), the phenotypes were correlated with the properties of the 30*S* subunits in extracts; this led to the proposal that the region between the *aroE* and *aroB* loci determines the structure of the 30*S* ribosome subunit (54, 104). Whether these genes determine the whole 30*S* subunit (16*S* RNA plus approximately 20 proteins) or only part of it is not known at this time. Similar clustering of the aminoglycoside resistance markers is found in *Bacillus* (179) and *Salmonella* (167).

The genetics of aminoglycoside resistance in *Bacillus* deserves special mention. Largely because of the work of Smith et al., finely detailed maps of the genes controlling antibiotic resistance have been obtained by transformation (179). The map in Fig. 2 shows that there is considerable scattering of antibiotic resistance markers in the region in question, inhibitors (putative) of the 30*S* (e.g., *str*, *spc*, *kan*, *neo*) and 50*S* (e.g., *ery*, *ole*) ribosomal subunit function being randomly clustered. Since the genes for 16*S* and 23*S* ribosomal RNA seem to fall outside the antiobiotic resistance markers, it appears that in *Bacillus* species the genes for ribosomal proteins are close to, but distinct from, the genes for ribosomal RNA. In *E. coli*, one of the regions homologous to 16*S* and 23*S* ribosomal RNA is very close to the *str* locus (32).

In other organisms, notably *Pneumococcus*, the genes conferring resistance to and dependence on aminoglycoside antibiotics showed less marked clustering (nonidentical alleles) and rather complicated cooperative effects were noted when different mutations were put together in the same strain (158, 163). In vitro polypeptide synthesis experiments have not yet been carried out with these mutants to determine whether ribosomes with altered properties are involved.

It should be apparent that there is considerable interest in studying mutations affecting a cell's response to aminoglycoside drugs, since these drugs provide a convenient way of studying ribosomal structure and function (particularly the 30*S* subunit) from a biochemical and genetic viewpoint. The bulk of the work along these lines is concerned with *E. coli*, and we have concentrated on studies with this organism (although the genetics of *Bacillus* species is well advanced). The reader should note that little progress has been made concerning the structural aspects of ribosomes and the relationship to mutational changes.

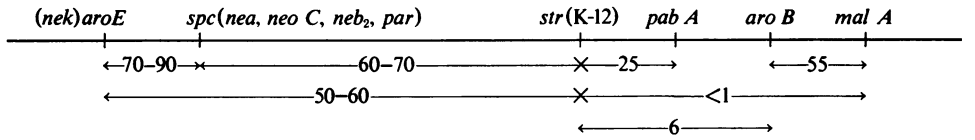

FIG. 2. *Streptomycin region on the E. coli chromosome. Approximate P1 cotransduction frequencies are given. These data have been compiled from the results of P. Anderson, L. Gorini, A. J. Pittard (personal communications), A. Bollen, and J. Davies (unpublished data).*

Mutagenesis. The frequency of spontaneous appearance of mutations to aminoglycoside resistance and dependence in bacteria is very low (138); *str*^{r} appears at a frequency of 10^{-9} and *str*^{d} at a frequency up to ten times lower. The spontaneous mutation rate of *spc*^{r} is the same or lower than *str*^{r} (36). The mutation rate from *str*^{d} to *str*Rd is of the order of 10^{-8}.

The reason for the apparently very low rate of mutation to aminoglycoside resistance and dependence is not clear. The low mutation frequencies may result from the fact that many of the mutations (in the ribosome) are lethal or from the fact that only specific base changes (amino acid substitutions) are permissible.

These mutation rates can be raised by the use of mutagens; the reagents nitrous acid (220), ethyl methane sulfonate (220), 2-aminopurine (J. Davies, *unpublished data*), 5-bromouridine (S. Brenner, *personal communication*), and nitrosoguanidine (174) raise the frequency of mutation to *str*^{r} and *spc*^{r} by a factor of 100 to 1,000. Verly et al. (220) emphasized the importance of the conditions of selection and described the optimal conditions for selection of mutants resistant to streptomycin; these workers claimed that all of the resistant mutants isolated after nitrous acid or ethyl methane sulfonate treatment were *str*^{d} and that such mutants were the result of multiple changes in DNA. Electrophoretic studies of the ribosomal proteins from a large number of mutants did not provide any information on the nature of mutations to resistance (E. C. Cox and J. G. Flaks, Federation Proc., p. 220, 1964).

The mutator gene discovered by Treffers et al. (206), which produces the specific adenine-thymine → cytosine-guanine transversion (230), is known to increase the appearance of *str*^{r} mutants 1,000-fold; interestingly, however, this mutator gene does not induce *spc*^{r} (E. C. Cox, *personal communication*; J. Davies, *unpublished data*). Thus, although there is good evidence that mutation in the *str* and *spc* loci are base substitutions (170*a*), there is apparently some base specificity in mutation to *spc*^{r}.

RTF. Infectious drug resistance mediated by RTF (R factors) has received considerable attention of late. This interest has been stimulated by the clinical importance of R factors and because of their use as simple models of gene replication. The R factors are independent linkage groups, similar to sex factors, carrying genes determining drug resistance together with information necessary for autonomous replication and transmission. Early experiments suggested that the R-factor carrying cells could inactivate drugs (129) or induce cell permeability to the drug (130). The drug resistance characters carried by R factors are listed in Table 3; it would not be surprising if more of such resistance characters appeared. Unlike chromosomal resistance to streptomycin and similar drugs, R-factor resistance is dominant to chromosomal streptomycin sensitivity.

Okamoto and Suzuki (145) were the first to report on the occurrence of specific inactivating enzymes from an R-factor strain; since this discovery, several inactivation reactions have been studied in detail (Table 3). It appears that enzymatic inactivation is the predominant mechanism of resistance; only tetracycline resistance is believed to result from a loss of permeability to the drug, although this has not been demonstrated conclusively. A full discussion of R factors and their genetics is beyond the scope of this article, but several reviews are available (127, 222).

INHIBITORS OF THE 50*S* SUBUNIT

At least two functions can be ascribed to the 50*S* subunit in protein synthesis: (i) it provides a site of attachment for peptidyl tRNA (64), the donor site; and (ii) it can participate in vitro in in the formation of the peptide bond (131).

Inhibition of 50*S* subunit function in vivo could, therefore, involve interference with these functions as well as with the movement of peptidyl tRNA and the ribosome relative to each other (translocation).

Table 3. *Resistance carried by R factors*

Drug	Mechanism of resistance	Reference
Chloramphenicol	Mono- and di-acetylation	159, 171, 173, 191, 213
Kanamycin	Phosphorylation and acetylation	94, 147, 211
Paromamine	Phosphorylation	94, 147, 211
Streptomycin	Adenylylation and phosphorylation	147, 192*a*, 211, 212, 227*a*; D. H. Smith, *personal communication*; Ozanne and Davies, *unpublished data*
Tetracycline	Permeability block (?)	213
Spectinomycin	Adenylylation	177, 178; Yamada and Davies, *unpublished data*
Neomycin	Phosphorylation	Ozanne and Davies, *unpublished data*

Subunit Localization Studies

The macrolide-, chloramphenicol-, and lincosaminide groups are chemically distinct classes of antibiotics whose site of action has been localized on the 50*S* subunit. Members of these antibiotic groups were found to bind exclusively to the 50*S* ribosomal subunit, and a requirement for this binding is the presence of ammonium or potassium ions (21, 214; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). A plateau level of binding is attained when an average of one antibiotic molecule is bound per 50*S* subunit; when a combination consisting of two of these antibiotics are present, competition for binding may be observed (21, 215-217; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). In studies by Das et al. (33) on chloramphenicol binding by *E. coli* ribosomes, two plateau levels were observed, one corresponding to one chloramphenicol molecule per ribosome and the second corresponding to two molecules per ribosome. Since saturation of the ribosomes with an average of one chloramphenicol molecule suffices to inhibit protein synthesis, the relationship between the second chloramphenicol molecule bound and the mechanism of inhibition by this antibiotic remains to be explained.

Certain characteristics of the competitive interaction were first described by Vazquez (215, 216) in a series of experiments in which intact bacteria, as well as partially purified ribosomes, were incubated with a combination of ^{14}C-chloramphenicol and another antibiotic in unlabeled form. A direct parallel was observed between the effects on uptake into intact cells and on the binding to purified ribosomes. The antibiotics which were found to compete with ^{14}C-chloramphenicol binding included macrolides (erythromycin, carbomycin, oleandomycin, spiramycin) and lincosaminides (lincomycin and celesticetin), which are inhibitors of the 50*S* subunit. Aminoglycosides (streptomycin, neomycin, and kanamycin) and tetracyclines (tetracycline and chlortetracycline), both known inhibitors of the 30*S* subunit, were without effect. Other experiments (21; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968) demonstrated a similar type of competitive interaction between erythromycin and ^{14}C-lincomycin. These data suggested that a competitive interaction for binding to ribosomes is typical of a number of classes of structurally (and possibly functionally) diverse antibiotics which are inhibitors of the 50*S* subunit.

This competitive interaction for binding to ribosomes has been remarkably consistent with 50*S* localization by at least another independent criterion, namely, the resistance of hybrid ribosomes reassociated from resistant and sensitive strains. By this method, the 50*S* subunit was also localized as the site of action of lincomycin and erythromycin (21; F. N. Chang, Ph.D Thesis, Univ. of Wisconsin, Madison, 1968). In view of the consistency of the technique with competition for ^{14}C-chloramphenicol binding, it seems safe to use this method in tentatively assigning the 50*S* subunit as the site of action of other antibiotics which inhibit ^{14}C-chloramphenicol binding. This method is especially useful when applied to antibiotics which are unavailable in labeled form or for which resistant mutants are not easily isolated.

Cundliffe and McQuillen (31) observed the inhibition of puromycin-dependent release of nascent peptide chains by chloramphenicol and erythromycin and the lack of such an effect by tetracycline, bottromycin, and pactamycin. The subunit specificity of the latter two antibiotics has not yet been determined. However, the inability of tetracycline to inhibit the puromycin reaction may be a general feature shared with other classes of 30*S* inhibitors.

Biochemical Effects and Mechanisms of Action

Some characteristics of protein-synthesizing systems inhibited by various antibiotics are discussed below.

Chloramphenicol. Chloramphenicol is exceptional since it is effective against both gram-positive and gram-negative organisms, whereas other inhibitors of the 50*S* subunit are more active against gram-positive organisms. Considering the large amount of literature on this antibiotic [*see* the recent review by Hahn (78)], remarkably little is known.

Recently, Monro and Marcker (132) studied a simplified peptide bond-synthesizing system which utilizes formyl-methionyl-ACCAAC (the terminal hexanucleotide fragment obtained from *N*-formyl methionyl tRNA by treatment with T_1 ribonuclease) as the peptidyl moiety, puromycin as the aminoacyl moiety, and 50*S* ribosomal subunits (131). The possible utilization of GTP, present as a contaminant, has not been ruled out. The reaction generates *N*-formyl methionyl puromycin, requires the presence of ethyl alcohol, and is inhibited by chloramphenicol or lincomycin, but not by erythromycin (33). These data were interpreted in terms of an inhibitory effect of chloramphenicol on the "peptidyl transferase," a structural component of the 50*S* subunit which directly forms the peptide bond. The inhibition of aminoacyl-tRNA binding observed earlier by Vazquez and Monro (219) was explained as a possible artifact resulting from actual inhibition of peptide bond formation which occurred, to an unspecified degree, in their binding assay.

Julian (89) made a detailed analysis of the oligolysine products synthesized in a cell-free poly A-directed protein synthesizing system inhibited by 400 μg of chloramphenicol per ml. Two classes of synthesized products were observed in the control experiment, a major peak of dilysine amounting to approximately 20% of the total labeled product, and a distribution of oligolysines ranging from lys_3 to lys_{11} with a peak at lys_6. In the presence of chloramphenicol (400 μg/ml), complete inhibition of lys_4 to lys_{11} synthesis and an increase in the level of lys_2 and lys_3 relative to higher lysine oligomers were observed; however, the absolute level of lys_2 and lys_3 synthesized did not vary significantly in either case. The relatively increased level of lys_2 and lys_3 could represent the effects of overall inhibition and the resultant synthesis of only small fragments, or the products of a phase of peptide bond synthesis which is resistant to chloramphenicol, or both. The possible contribution of proteolytic activity in the crude extract used to the high levels of dilysine found was not assessed. The synthesis of oligolysines directed by oligoadenylates endogenously synthesized [from adenosine triphosphate (ATP) by poly A polymerase] could also, in principle, generate the type of data reported by Julian (89). In any case, one is clearly dealing with a heterogeneous (probably bimodal) collection of polynucleotide messengers and it should not be surprising if the distribution of products of such a system deviate from an expected random distribution of chain lengths. Assuming that the observed dilysine synthesis utilizes oligoadenylates as messenger and is resistant to chloramphenicol, it would be necessary to consider a class of mechanisms which form a single peptide bond but do not translocate the product so as to polymerize additional residues.

As a possible test to distinguish between primary inhibition of the peptide bond forming step and some other reaction involving movement of aminoacyl or peptidyl tRNA, it would be of interest to compare the antibiotic sensitivity of oligoadenylate-directed systems (or other suitably devised systems) which synthesize di- and tripeptide, respectively.

Another striking feature of chloramphenicol action is its relative effectiveness as an inhibitor of poly A- and poly C-directed polylysine and polyproline synthesis, respectively, as compared with poly U-directed polyphenylalanine synthesis, which was found to be relatively resistant to the action of this drug by Speyer et al. (183). This pattern of differential inhibition of various homopeptide synthesizing systems has been observed for other known 50*S* inhibitors and suggests a possible common mechanism of action (216).

Erythromycin. Cundliffe and McQuillen (31) studied the puromycin-dependent release of nascent peptide chains, in protoplasts of *B. megaterium*, in the presence of different antibiotics. Their experimental design consisted of blocking protein synthesis with various ribosome inhibitors and then testing the ability of the system to form a single peptide bond between the growing peptide chain and puromycin. Their observations are summarized as follows: when a sequence of erythromycin and puromycin was used, no peptidyl puromycin was formed; when a sequence of chloramphenicol and puromycin was used, no peptidyl puromycin was formed; when a sequence of tetracycline, erythromycin and puromycin was used, peptidyl puromycin was formed; when a sequence of tetracycline, chloramphenicol, and puromycin was used, no peptidyl puromycin was formed.

It was concluded from these observations that chloramphenicol inhibits a putative "peptidyl

polymerase," whereas erythromycin inhibits a "translocase"; i.e., a factor which promotes the movement of the peptidyl tRNA, elongated by a single residue, from the acceptor site to the donor site. According to the model proposed, peptidyl tRNA, in the presence of erythromycin would remain stuck in the acceptor site after having been elongated by a single aminoacyl residue. This, in turn, would prevent access of puromycin to that portion of the 50*S* subunit corresponding to the aminoacyl site. In the presence of tetracycline, however, peptidyl and aminoacyl tRNA, but not puromycin binding at the aminoacyl site, would be inhibited. Puromycin could then react to form peptidyl puromycin.

Formylmethionyl puromycin formation from formylmethionyl tRNA, puromycin, 70*S* ribosomes, and AUG (or 50*S* subunits in the absence of messenger) is unaffected by erythromycin but sensitive to chloramphenicol (133; F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). This fact is consistent with a model in which formylmethionyl $tRNA^F$ has access to the peptidyl site and leaves the aminoacyl site accessible to puromycin. Other studies on peptide bond formation, however, have shown that this reaction is sensitive to carbomycin, a macrolide antibiotic (99% inhibition at 10^{-4} M; 133). There is, thus, an apparent inconsistency. Since formylmethionyl puromycin synthesis would not be expected to involve a translocation step, we are forced to conclude that macrolide antibiotics, despite their chemical similarity, do not all have a common mode of action.

Tanaka et al. (193) observed a preponderance of di- and trilysine in polylysine-synthesizing cell-free extracts inhibited by erythromycin. They concluded that erythromycin inhibits the ability of ribosomes to synthesize highly polymerized lysine homopeptides but that such inhibited ribosomes still retain their ability to synthesize (mechanism unstated) small peptides such as di- and trilysine. As in the case of the chloramphenicol-inhibited system, it cannot be ascertained whether the level of small peptide synthesis conforms to the statistical distribution expected from an overall inhibition of all peptide bonds or whether the small peptides represent a "core" of antibiotic-resistant synthesis. It might be expected that erythromycin would have no effect on dilysine synthesis in such a system if the first lysyl tRNA entered the peptidyl site directly, because a translocation reaction would only be required in the synthesis of tri- and higher oligolysines. This question might be resolved by the use of an oligoadenylate as mRNA, as outlined above.

Lincomycin. The inhibition of ^{14}C-chloramphenicol binding by lincosaminide antibiotics was noted by Vazquez (215). Chang and Weisblum (21) and Chang (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968) subsequently demonstrated directly the binding of ^{14}C-lincomycin exclusively to the 50*S* subunit, some requirements for this binding reaction, and its reversal by erythromycin. In an attempt to characterize the inhibition of protein synthesis, it was observed that the binding of phenylalanyl tRNA, tested according to the method of Nirenberg and Leder (141), could be inhibited by chloramphenicol, erythromycin, and lincomycin (20, 219). Further investigation, however, revealed that a significant level of polymerization had occurred despite extensive washing of the ribosomes to remove bound factors and that the apparent inhibition of binding could be interpreted in terms of an inhibition of peptide-bond formation in the binding assay. The inhibition of peptide bond synthesis per se however, cannot be explained by the apparent inhibitory effects of chloramphcicol, lincomycin, and erythromycin on the aminoacyl tRNA binding reaction, since there are other instances in which antibiotics which inhibit protein synthesis have no effect on, or may even stimulate, aminoacyl tRNA binding, e.g., amicetin (219).

The range of lincomycin action with respect to the peptide bonds affected differs from that of erythromycin. Cell-free systems which synthesize formylmethionyl puromycin or polylysyl puromycin (164) enable us to isolate, for study, the formation of a single peptide bond. Whereas formylmethionyl puromycin synthesis is unaffected by erythromycin, lincomycin exerts a strong inhibitory effect on this reaction (133). Although erythromycin does not inhibit the reaction, it can reverse the inhibition produced by lincomycin (F. N. Chang, Thesis, Univ. of Wisconsin, 1968), indicating that erythromycin indeed interacts with the ribosome. On the basis of the data presently available, we cannot conclude that the mode of action of lincomycin differs from that of chloramphenicol in a significant qualitatively different way.

Although the chemical nature of the binding site(s) for these antibiotics is not yet known, some clues are available from structure-function relationships. Certain chemical modifications of the lincomycin molecule which tend to make it more lipophilic also increase its antibacterial activity. Thus, derivatives such as 7-chloro- or *N*-ethyl-, as well as those containing pentyl or butyl substituents on the prolyl moiety, show greater antibacterial activity against gram-positive organisms than the parent compound (123*a*). A detailed description of the antibacterial effects of the parent compound was reported by Lewis et al. (111).

Two classes of mechanisms may account for

the observed increase in antibacterial activity: first, increased solubility in the lipid of the cell membrane which might facilitate transport, and, second, increased affinity for a (partly) lipophilic binding site which could result in greater effectiveness at the site of action.

Increased lipid solubility may also account for the effectiveness of 7-chloro 7-deoxy lincomycin against *Plasmodium bergheii* as well as for the increased activity of several other 7-chlorinated derivatives (110) against gram-negative organisms. In plasmodia, this might involve increased solubility in lipids which are present in the cell membrane as well as those found in mitochondria. The inhibition of mitochondrial protein synthesis by antibiotic inhibitors of bacterial ribosomes and its implications are discussed further below. In the case of gram-negative organisms, the lipopolysaccharide and lipoprotein portions of the cell wall may also represent barriers which can be penetrated more effectively by antibiotics with greater lipid solubility.

Structure-function relationships in the chloramphenicol series have been examined with respect to both the nitrophenol and dichloroacetamido substituents. Coutsogeorgopoulos (27, 28) postulated that chloramphenicol is an analogue of the acceptor end of aminoacyl tRNA in which the dichloroacetamido substituent corresponds to the amino acid. He studied a series of chloramphenicol analogues in which the acetamido group was replaced by various aminoacyl moieties, namely, phenylalanine, glycine, leucine, or *p*-methoxyphenylalanine In one series of experiments, poly U-directed phenylalanine incorporation was used as the assay system. The phenylalanine and glycine analogues gave the weakest inhibition (2% at a concentration of 10^{-3} M), whereas the other chloramphenicol analogues were more effective and inhibited to an extent of 25% at this level. This series of experiments provided some useful information on the side group requirements for antibiotic action. However, in terms of the model proposed for chloramphenicol action, the evidence presented is at best suggestive.

Cammarata (16) studied the antibacterial effects of various chloramphenicol analogues differing in the nitrophenol substituent. Inhibition rate constants of the various analogues (intact cells of *E. coli*) were compared with the polarizability (i.e., ability to induce a net electrical dipole moment) of the benzene ring by different substituents in the *para* position replacing the nitro group. A strong positive correlation between the coefficient of polarizability and the antibacterial effect was noted. Differences in antibacterial effects could reflect relative differences in membrane permeability, relative differences in affinity for the antibiotic binding site, or relative differences in the efficacy of the antibiotic once it is bound. These data cannot be clearly interpreted unless experimental results obtained from cell-free systems are also taken into consideration.

Genetic Studies

Phenotypic aspects. Antibiotic binding studies discussed above indicate that chloramphenicol, erythromycin, and lincomycin bind to ribosomes in a mutually exclusive fashion and in a 1:1 ratio with respect to the ribosome. In a sense, therefore, a ribosome to which erythromycin is bound can be said to be "virtually" resistant to lincomycin. Inhibition of protein synthesis under these conditions might still be seen, but it would be due to the antibiotic which is more strongly bound. In cell-free systems derived from a sensitive strain of *B. stearothermophilus* and in which inhibition of the synthesis of defined, isolated peptide bonds could be studied, an apparent total resistance to lincomycin was observed in the presence of erythromycin (F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison 1968). Thus, the inhibition of *N*-formyl methionyl puromycin synthesis by lincomycin could be reversed by erythromycin. In sensitive organisms, this situation may not occur for all peptide bonds, so that the net result of a combination would still be an inhibition of protein synthesis.

The implications of this type of resistance are of special importance in the effective clinical utilization of these antibiotics. On the basis of these considerations, the simultaneous use of a competitive pair of 50*S* inhibitors would be expected to result in the therapeutic effects of at most one drug but the combined risks of both drugs. A possible exception to this consideration, however, is the synergistic effect of a combination consisting of a streptogramin A-type and B-type antibiotic (47, 218).

A mutant of *S. aureus* resistant to erythromycin but sensitive to spiramycin, another macrolide, was described by Chabbert (18, 19). Because resistance did not extend to other macrolides such as oleandomycin and spiramycin, this pattern was referred to by Garrod (62) as resistance of the "dissociated" type. Studies on this erythromycin-resistant mutant revealed that resistance to spiramycin could be induced when the mutant was cultivated in the presence of erythromycin at levels to which it had acquired resistance or even at much lower, subinhibitory levels (19, 62).

In further studies on (dissociated) erythromycin-resistant organisms, Barber and Waterworth (9) and Griffith et al. (77) observed that

such strains, when grown in the presence of erythromycin, displayed conditional resistance to lincomycin as well. Further studies in our laboratory (B. Weisblum, *unpublished data*) have shown that conditional resistance can also be expressed toward streptogramin B-type antibiotics but not toward streptogramin A-type antibiotics, chloramphenicol, amicetin, sparsomycin, or aminoglycosides.

By analogy with RTF-carrying strains, the fact that dissociated resistance has only been observed in strains isolated from patients suggests a possible episomal nature of the genetic determinants for this type of resistance. Griffith et al. (77) searched for possible conversion products of lincomycin in strains of *S. aureus*, selected for erythromycin resistance, which displayed dissociated resistance to lincomycin. No biologically active conversion products of lincomycin, erythromycin, or lincomycin-erythromycin interaction products were detected by the one-dimensional chromatographic method used, and bioassay of the broth medium did not detect destruction of antibiotic activity.

A possible mechanism for this type of conditional resistance was suggested by competitive interactions on the ribosome, between known 50*S* inhibitors and, in particular, between erythromycin and lincomycin for binding to *B. stearothermophilus* (sensitive) ribosomes (21). It was proposed that dissociated resistance involving erythromycin and other 50*S* inhibitors was due to an alteration in the ribosome and that it resulted in ribosomes having decrased affinity for (or decreased sensitivity to) erythromycin. Concentrations of erythromycin to which the mutant acquired resistance, according to the model, would be ineffective in inhibiting protein synthesis on these ribosomes but would still be capable of inhibiting lincomycin (or spiramycin) binding to the ribosome. In view of the ability of erythromycin to inhibit chloramphenicol binding to the ribosome, it is surprising that conditional resistance to chloramphenicol in erythromycin-resistant strains (grown in the presence of erythromycin) has not been reported.

Other patterns of resistance have been described. Certain strains of *S. aureus* which show resistance to erythromycin, oleandomycin, and spiramycin have been isolated. Only this type of resistant mutant, but not the dissociated type, can be obtained by in vitro selection methods (62). The biochemical basis for this type of mutation is more easily understood (than that for dissociated resistance) in terms of an alteration in the receptor which decreases its affinity for a whole class of chemically similar antibiotics. For obvious reasons, the term "double" resistance utilized by Garod (62) inaccurately describes the phenomenon observed, since resistance can extend to numerous distinct but chemically similar antibiotics. The term "cross-resistance" would probably describe this phenomenon more accurately.

Another type of apparent cross-resistance is observed in mutants which have developed generalized impermeability to many different antibiotics, regardless of their chemical structures or modes of action (160). These forms of resistance are relatively uninteresting, however, in terms of ribosomal mechanisms of antibiotic resistance.

The (standard) strain of *B. stearothermophilus* (strain 1503) which we used is normally sensitive to concentrations of erythromycin greater than 1 μg/ml. A mutant resistant to 10 μg of erythromycin per ml was selected (without mutagenic treatment) and was found to have acquired at least a 10-fold increase in resistance to chloramphenicol as well (F. N. Chang, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1968). Ribosomes obtained from this strain were found to have low activity in cell-free protein synthesis and to be unable to bind ^{14}C-erythromycin, ^{14}C-chloramphenicol, or ^{14}C-lincomycin when tested by the membrane filter method. In comparison with the wild type, the 50*S* subunits obtained from the mutant were found to be relatively unstable in CsCl under conditions used for preparing subparticles and split proteins (203, 204). The 50*S* subunits from the mutant strain were found to yield appreciable amounts of denser subparticles which presumably contain less protein per unit of ribosomal RNA.

Genotypic aspects. Various patterns of resistance and sensitivity in *E. coli* toward lincomycin and erythromycin were recently reported by Apirion (6). *E. coli* cells are naturally resistant to between 300 and 400 μg of erythromycin or lincomycin per ml. Mutants sensitive to lower levels or resistant to higher levels of the antibiotics were obtained. On the basis of mapping data and antibiotic response profiles, three loci were defined, called "*lir*, *lin*, and *ery*"; these loci correspond, respectively, to lincomycin and/or erythromycin sensitivity, lincomycin resistance, and erythromycin-resistance. Flaks [quoted by Taylor and Trotter (202)] also found loci which he named "*lin*" and "*ery*" and which correspond to lincomycin and erythromycin sensitivity, respectively. They map close to Apirion's lir_{37}. Recently, Krembel and Apirion (96) observed an altered 50*S* ribosomal protein associated with a "*lir*" mutant, but the structures or functions related to the other genetic loci have not yet been defined. Their phenotypic properties and map positions are summarized in Table 4.

The lack of unanimity in naming the various

TABLE 4. *Summary of genetic loci in E. coli which affect sensitivity or resistance to erythromycin and lincomycin*[a]

Locus	Maximal tolerable concn[b]		Isolated by selection for	Cross effects	Map position
	Erythromycin	Lincomycin			
	μg	μg			
lir	<200	<150	Lincomycin sensitivity	Erythromycin sensitivity	10–15
lin	800	>1,500	Lincomycin resistance	Erythromycin resistance	10–40
ery	>1,000	400	Erythromycin resistance	None	5–15
lir_{37}	300	200–300	Lincomycin sensitivity	Erythromycin sensitivity	60–65

[a] *See* reference 6.

[b] The natural resistance of the strain employed was 400 μg/ml for both erythromycin and lincomycin.

antibiotic sensitivity and resistance loci is confusing. It would be desirable for workers in this area to agree on a common nomenclature for the loci involved in sensitivity or resistance to antibiotic inhibitors of the 50*S* subunit.

RTF. Some properties of RTF were discussed above. Certain RTF are also capable of inactivating inhibitors of 50*S* function as well as antibiotics, such as sulfonamides, which have no known direct inhibitory effects on ribosomal function.

In the case of chloramphenicol, acetylation dependent on acetyl coenzyme A was found to be the mode of expression of certain episomal elements from *Enterobacteriaceae* which confer resistance to chloramphenicol (145). A detailed analysis of the acetylated products was reported by Shaw and co-workers (171–173), who observed that chloramphenicol is converted to 3-acetoxy and 1,3-diacetoxy chloramphenicol by certain resistant strains of both *S. aureus* and *E. coli*.

Permeability mutants. E. coli cells can acquire resistance to puromycin, tetracyclines, and chloramphenicol in a single mutational step (160). Colonies grown from such mutants are mucoid and appear to arise from mutations at the "*capR*" locus, which has been implicated in capsular polysaccharide synthesis. Permeability type mutants resistant to inhibitors other than chloramphenicol can presumably also be isolated by a similar selection procedure.

LESS EXTENSIVELY CHARACTERIZED INHIBITORS

The antibiotics which are discussed in this section have figured less prominently in studies involving the inhibition of bacterial protein synthesis. Although their inhibitory action has been less extensively characterized than that of the antibiotics discussed above, they are no less interesting. The compounds on which we will focus our attention in this section are streptogramins, sparsomycin, amicetin, blasticidin, fusidic acid, rifamycins, pactamycin and bottromycin, viomycin, and bryamycin and micrococcin.

Streptogramins

The name used for this heading refers, collectively, to the group of antibiotics synthesized by *Streptomyces graminofaciens.* These antibiotics are isolated as a complex mixture which can be resolved into at least two chemically distinct classes referred to as "A" and "B."

Other streptogramin-type antibiotic complexes include the osterogrycin, mikamycin, staphylomycin, and synergistin families. The properties of these antibiotics were studied by Vazquez (215–217), Vazquez and Monro (219), and Monro and Vazquez (133). For a recent review *see* Vazquez (218).

For the streptogramins, Vazquez reported that the A component inhibits ^{14}C-chloramphenicol binding to *E. coli* ribosomes, whereas the *B* component does not (215). However, it was later noted that viridogrisein (a B-type component) could inhibit the binding of spiramycin III (a macrolide) to *E. coli* ribosomes (217). These data imply that the 50*S* subunit is the site of action of both streptogramins A and B.

In previous studies, Vazquez and Monro (219) initially observed that the streptogramin B group did not inhibit ^{14}C-chloramphenicol binding to *E. coli* or *B. megaterium* ribosomes and, in addition, produced an apparent stimulation of the extent of aminoacyl tRNA binding to 70*S* ribosomes. Since a similar stimulation of aminoacyl tRNA binding to 30*S* subunits was found to parallel the effect on 70*S* ribosomes, the 30*S* subunit was identified as the site of action of streptogramin B. Although the basis for this phenomenon is not well understood, the failure of streptogramin B to compete with chloramphenicol for binding to *E. coli* ribosomes was probably due to a relatively weaker binding of chloramphenicol by this subunit.

Investigations in our laboratory (F. N. Chang,

Table 5. *Summary of the effects of several test antibiotics on ^{14}C-chloramphenicol and ^{14}C-spiramycin binding to ribosomes*

Antibiotic	Inhibition of fragment reaction in *E. coli*[a]	Inhibition of ^{14}C-chloramphenicol binding to *B. megaterium* ribosomes[b]	Inhibition of ^{14}C-chloramphenicol binding to *E. coli* ribosomes[c]	Inhibition of ^{14}C-spiramycin binding to *E. coli* ribosomes[d]	Inhibition of ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes[e]	Concn of antibiotic used in *B. stearothermophilus* studies[e]
Streptogramin A family	Yes	Yes	Yes	Yes	Yes (99%)	2×10^{-4} M (vernamycin A)
Streptogramin B family	No	No	No	Yes	Yes (83%)	2×10^{-4} M (vernamycin B)
Sparsomycin	Yes	Not done	No	Not done	Yes (85%)	1×10^{-3} M
Amicetin	Yes	Not done	No	Not done	Yes (60%)	2×10^{-4} M
Blasticidin	Not done	Not done	Not done	Not done	Yes (60%)	2×10^{-4} M
Fusidic acid	Not done	Not done	Not done	Not done	No (19%)	2×10^{-3} M
Rifamycin	Not done	Not done	Not done	Not done	No (13%)	1×10^{-3} M

[a] *See* reference 133.
[b] *See* reference 215.
[c] *See* reference 219.
[d] *See* reference 217.
[e] Based on F. N. Chang, C. Siddhikol, and B. Weisblum, *unpublished data*.

C. Siddhikol, and B. Weisblum, *unpublished data*; summarized in Table 5) have revealed that the binding of ^{14}C-chloramphenicol to *B. stearothermophilus* ribosomes is inhibited both by members of the A group (streptogramin A and vernamycin A) and the B group (vernamycin B complex and viridogrisein).

Thus far, ribosomes from gram-negative organisms are equally or less sensitive than ribosomes from gram-positive organisms, as a general rule. It would be expected, therefore, that ribosomes from a gram-positive organism would be able to interact with a much wider range of antibiotics and would be better suited for the assay of chloramphenicol binding inhibition.

Ennis (47) characterized certain features of the inhibition of ribosomes produced by the antibiotics PA 114A and PA 114B (synergistin A and B, respectively) which are members of the streptogramin family. The A component was capable of irreversibly inhibiting the function of the 50*S* subunit of ribosomes obtained from *E. coli*. Thus, when 30*S* and 50*S* subunits were exposed to PA 114A and PA 114B, respectively, and were dialyzed against antibiotic-free buffer, only the 50*S* subunits were inactivated. We cannot rule out that a reversible but physiologically significant reaction occurs with the 30*S* subunit. However, the tentative designation of the 50*S* subunit as the site of action of the A component is in agreement with Vazquez' data (215) concerning the effectiveness of this class of antibiotic in reversing ^{14}C-chloramphenicol binding to the ribosome. Lack of positive data concerning subunit inactivation by PA 114B prevented subunit localization of the site of action of this antibiotic.

Inhibition of poly U-directed ^{14}C-phenylalanyl tRNA binding to *E. coli* ribosomes was observed for both PA 114A and PA 114B. However, the ribosomes used were not purified through buffers containing high salt concentrations, and it must be assumed, until proven otherwise, that part of the observed inhibition was the result of peptide bond formation.

Sparsomycin

Sparsomycin was studied by Slecta (176) and by Jayaraman and Goldberg (88). This antibiotic has a very broad spectrum of activity and is toxic to both gram-positive and gram-negative organisms as well as to eucaryotic cells. Considered together with other data, the fact that sparsomycin inhibits both bacterial protein synthesis and hemoglobin synthesis in cell-free extracts from reticulocytes (24) implies that sparsomycin is an inhibitor of both 70*S*- and 80*S*-type ribosomes. Goldberg and Mitsugi (65) proposed that sparsomycin directly inhibits the actual peptide bond forming step. This is consistent with the observations of Monro and Vazquez (133), who found that sparsomycin could inhibit the fragment reaction. The fact that sparsomycin can interfere with ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes (Table 5) suggests that part of the antibiotic interacts with the 50*S* subunit rather than with associated soluble factors.

Amicetin

The effects of amicetin in intact cells and in a cell-free system from *E. coli* were studied by Block and Coutsogeorgopoulos (10). Inhibition of growth of *Streptococcus faecalis* was found to require only one-tenth the concentration of amicetin needed to inhibit *E. coli*. Amicetin does not inhibit ^{14}C-chloramphenicol binding to *E. coli* ribosomes (215) but does inhibit the fragment reaction (133) with *E. coli* ribosomes.

In our own studies (Table 5), the binding of ^{14}C-chloramphenicol to *B. stearothermophilus* ribosomes was inhibited by amicetin. In addition, ribosomes from *B. stearothermophilus* were found to be more sensitive than ribosomes from *E. coli* by a factor of approximately 2.5. The higher level of sensitivity in *E. coli-B. stearothermophilus* hybrid ribosome combinations was found to be associated with the preparation containing 50*S* subunits from *B. stearothermophilus*.

As a possible clue to the mode of action of amicetin, Block and Coutsogeorgopoulos (10) considered the structural similarities in a group of antibiotics which they defined as "amino acylaminonucleoside" antibiotics. These include puromycin, gougerotin, homocitrullyl aminoadenosine, blasticidin S, and amicetin. They share the common structural features of a base, an amino sugar, and an aminoacyl moiety. In addition, it was postulated that a particular conformation of the chloramphenicol molecule might simulate an amino acylaminonucleoside structure. In view of the similarity of these antibiotics to the acceptor terminus of tRNA, it was proposed that they interact with that portion of the ribosome which recognizes the aminoacyl adenosine moiety of tRNA. Many of the available data are not in conflict with this hypothesis, but a more direct demonstration would be desirable.

Blasticidin S

Blasticidin S inhibits the growth of gram-positive and gram-negative bacteria as well as fungi. Some of the inhibitory effects of this antibiotic have been reviewed by Misato (128). The fact that blasticidin has a very broad spectrum suggests that it may be an inhibitor of both 70*S* and 80*S* ribosomes. As discussed above, Block and Coutsogeorgopoulos (10) emphasized the possible structural similarity between blasticidin and puromycin.

Yamaguchi and Tanaka (228) studied the inhibitory effects of blasticidin S on cell-free protein synthesis in *E. coli* extracts. The puromycin-dependent release of nascent peptide chains was inhibited by blasticidin S. In our laboratory (Table 5), blasticidin was found to inhibit ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes; this finding is consistent with the localization of the action of this antibiotic on the 50*S* subunit

Fusidic Acid

Fusidic acid is unique in that it has a steroid-like structure. It inhibits protein synthesis in intact cells of *S. aureus* but has no effect on intact *E. coli* cells (80). In cell-free extracts from *E. coli*, *B. stearothermophilus*, and yeast, protein synthesis is inhibited by fusidic acid. Tanaka et al. (195) recently observed that fusidic acid can inhibit the G factor from *E. coli*. They noted that the release of ^{32}P from ^{32}P-γ-labeled GTP was inhibited by fusidic acid. In earlier studies by Tanaka et al. (199), fusidic acid failed to inhibit puromycin-dependent release of nascent peptide chains from the ribosome but it did inhibit chloramphenicol binding.

The findings of our laboratory (F. N. Chang, C. Siddhikol, and B. Weisblum, *unpublished data*) are in partial agreement with these observations. Thus, in a guanosine triphosphatase reaction dependent on G-factor from *E. coli*, ribosomes from *B. stearothermophilus*, and ^{14}C-GTP, GTP degradation to guanosine diphosphate was inhibited at 10^{-4} M fusidic acid. However, no inhibition of ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes was found. If G-factor-dependent hydrolysis of GTP drives the translocation reaction, as suggested by Conway and Lipmann (26), fusidic acid may be a unique inhibitor of this reaction.

Since the guanosine triphosphatase reaction only proceeds in the presence of G-factor plus 70*S* ribosomes (but not isolated 30*S* or 50*S* subunits), both subunits appear to contribute to this reaction. In view of these complex requirements, the localization of fusidic acid action remains to be more precisely defined.

Rifamycins

The rifamycins are a group of structurally similar complex macrocyclic antibiotics. There has been some disagreement as to whether they primarily affect RNA synthesis or protein synthesis. Their mode of action has been reviewed by Frontali and Tecce (58). Evidence was presented that cell-free protein synthesis was inhibited by these antibiotics. It was also noted that preincubation of ribosomes with poly U before the addition of rifamycins could partially protect these ribosomes against the inhibitory action of the antibiotics; therefore, inhibition of mRNA binding to ribosomes was proposed as the mode of action of these antibiotics.

Sippel and Hartmann (175) and Umezawa et al. (210) recently presented compelling evidence that rifamycin is an inhibitor of RNA synthesis and that it belongs to a class of inhibitors which interact with the RNA polymerase molecule rather than with the DNA template. They observed that a DNA-dependent RNA-polymerization reaction, once initiated, was insensitive to inhibition by rifamycin. However, when rifamycin was preincubated with RNA polymerase before the addition of triphosphates and the DNA template, the reaction was almost completely abolished. Certain features of the initiation of RNA synthesis have been described by Maitra et al. (121). In view of the fact that resistance to rifampicin in *E. coli* has been found in association with an altered RNA polymerase which is unable to bind the drug (223*a*), it is hard to see how any of the reported effects of rifamycin on protein synthesis bear any relation to the mode of action of this drug.

In our studies, (Table 5) rifamycin did not inhibit ^{14}C-chloramphenicol binding to *B. stearothermophilus* ribosomes, nor did it inhibit poly U-directed phenylalanine incorporation in a cell-free system prepared from this organism.

Pactamycin and Bottromycin

Inhibition of protein synthesis by pactamycin was studied by Colombo et al. (24) and by Felicetti et al. (51). Pactamycin appears to have a very broad spectrum of action, affecting mammalian cells as well as gram-positive and gram-negative bacteria. In the Studies of Felicetti et al. (51), reticulocyte ribosomes were inactivated by incubation with pactamycin, and a shift from polysomes to single ribosomes was observed.

The inhibitory effects of bottromycin on intact cells and extracts from *E. coli* were studied by Tanaka et al. (197). They observed an inhibition of the transfer of aminoacyl tRNA into protein without concomitant inhibition of amino acid attachment to tRNA. Bottromycin did not inhibit puromycin-dependent release of nascent peptide chains.

In the studies of Cundliffe and McQuillen (31), pactamycin, bottromycin, and tetracyclines did not inhibit puromycin-dependent nascent peptide chain release, whereas chloramphenicol, erythromycin, and sparsomycin, which act on the 50*S* subunit, did inhibit the reaction. These data suggest the possibility that pactamycin and bottromycin, when acting on bacterial ribosomes, inhibit a function of the 30*S* subunit in accordance with the mechanism discussed above. Consistent with these observations, but only suggestive in terms of the localization of the site of action of these antibiotics on the 30*S* subunit, is the finding from our laboratory (C. Siddhikol and B. Weisblum, *unpublished data*) that pactamycin and bottromycin do not inhibit the binding of ^{14}C-chloramphenicol to *B. stearothermophilus* ribosomes.

Viomycin

Viomycin is a strongly basic polypeptide antibiotic which is very active against mycobacteria and only slightly active against gram-negative organisms. Tsukamura found it to be an effective inhibitor of protein synthesis in *Mycobacterium avium* (207). White and White showed that it was bactericidal to *E. coli* at high concentration and synergistic with puromycin (224). Subsequent experiments with polypeptide synthesis in *E. coli* extracts showed that viomycin was a very powerful inhibitor of protein synthesis; it caused little or no misreading (40, 194). Although viomycin almost certainly inhibits protein synthesis at the ribosome level, the lack of sensitive strains and corresponding resistant mutants has discouraged further study.

Bryamycin and Micrococcin

Bryamycin and micrococcin were characterized as inhibitors of protein synthesis by S. Neubort and J. Marmur (*personal communication*) on the basis of studies involving ^{14}C-amino acid, uracil, and thymidine incorporation by intact cells. In genetic studies of *B. subtilis* by Dubnau et al. (45), loci concerned with resistance to these antibiotics were mapped and located near the *str*^{r} locus.

Effects of 70*S* Ribosome Inhibitors in Eucaryotic Cells

Although the spectrum of cytotoxic action of the antibiotics discussed above is concerned primarily with procaryotic cells, even those which are not inhibitors of 80*S* ribosome function have quite marked effects on eucaryotic organisms. The selective bleaching effect of streptomycin on chloroplasts in *Chlamydomonas* has been known for some time and is well documented (155, 165). Erythromycin exerts a similar inhibitory effect on chloroplasts in *Euglena* (46). Streptomycin has been reported to interfere with the production of antibodies in vitro, producing antibodies with altered serological activity (97). However, it is not clear whether this effect is due to a direct action on protein synthesis or to the formation of a streptomycin-antigen complex (98). Inhibition of the growth of cultured animal cells by streptomycin (135), which occurs only in the presence of cysteine (136), may be due to the toxicity of the formed cysteine-streptomycin complexes (137)

and not to streptomycin alone. Dihydrostreptomycin, which does not form such a compound with cysteine, is inactive in the presence or absence of cysteine. Neomycin can inhibit cell-free polypeptide synthesis in chick embryo extracts but no translation errors have been detected (R. Soeiro, *personal communication*).

Yeast cells grown in the presence of chloramphenicol, erythromycin, or lincomycin become depleted of cytochromes *a*, a_3, *b*, and c_3 (86). Data concerning inhibitory effects of a large series of antibiotics on yeast cytochromes (22) and on chlorophyll levels in *Euglena* (113) have been reported. Linnane et al. (112) compared the sensitivity of cell-free protein synthesis to various antibiotics with mitochondrial suspensions or cytoplasmic ribosomes. Mitochondrial protein synthesis was sensitive to chloramphenicol, macrolides, and lincomycin, but resistant to cycloheximide. Protein synthesis on cytoplasmic extramitochondrial ribosomes, however, was resistant to chloramphenicol, macrolides, and lincomycin, but sensitive to cycloheximide.

The significance of these results becomes more apparent in view of the fact that cytoplasmic ribosomes are of the 80*S* type (11, 100), whereas mitochondrial (100) and chloroplast (11) ribosomes are of the 70*S* type. The toxic effects of bacterial ribosome inhibitors in eucaryotic cells could be attributable, at least in part, to an inhibition of the bacterial type 70*S* ribosomes found in chloroplasts and mitochondria.

The implications of these data for theories concerning possible common origins of protein synthesizing systems having similar ionic requirements and antibiotic sensitivities are obvious. The intriguing possibility that certain organelles may have arisen by parasitic or symbiotic infestation of one cell type by another is consistent with comparative antibiotic sensitivity studies such as these. In this context, the nucleus is another cellular compartment in which proteins are synthesized; it would also be of interest to characterize nuclear protein synthesis with respect to its comparative antibiotic sensitivity.

Other inhibitory effects of antibiotics in mammalian systems, attributable to inhibition of protein synthesis, have been reported. Inhibition of antibody formation after treatment with chloramphenicol (2, 140) has also been described. Very recently, deficiency of cytochrome *c* reductase was reported in rat heart cells cultured in vitro in the presence of chloramphenicol (99). Cloning efficiency of a cell line derived from mouse bone marrow was found to be decreased by 98% in the presence of 25 μg of chloramphenicol per ml (44). This only represents a partial listing of the toxic effects in mammals produced by antibiotics which inhibit bacterial protein synthesis. Preferential inhibition of certain tissues may reflect preferential solubility of these antibiotics in the lipids of the cell type involved.

Toxic effects of these antibiotics have also been noted in man. It would be expected that untoward effects might be most marked in those cell lines which have the most rapid turnover rate. In man, these include the bone marrow and the intestinal epithelium.

Clinical features of the toxic effects of chloramphenicol on the bone marrow were reviewed by Yunis and Bloomberg (232). They discussed two classes of toxic reactions. (i) An immediate decrease in erythroid elements resulting primarily in anemia; this effect is dose-related and is reversible by cessation of chloramphenicol administration. (ii) Depression of the marrow involving all cellular elements; this effect occurs 2 to 8 weeks after cessation of chloramphenicol administration and is neither dose-related nor reversible by cessation of the drug. It is usually fatal.

Effects on the intestinal epithelium have been described in relation to a clinical syndrome known as "pseudomembranous enterocolitis," which is characterized by a loss of regenerative function of the intestinal epithelium and by necrosis. In a retrospective study of a series of 17 unselected cases, reported by Hartmann and Angevine (79), 12 cases had received tetracyclines before the onset of symptoms. Other toxic effects of tetracyclines on mammalian protein synthesis have been observed (140, 231). Various other etiological models can be proposed for the observed toxicity of tetracyclines; however, in view of what has been learned about the inhibitory effects of antibiotics in eucaryotic cells, the conjecture offered above warrants further consideration.

The inhibitory effects of these antibiotics in eucaryotic cells may actually be utilized in designing drugs against certain parasites. As was mentioned above, 7-chloro-7-deoxylincomycin is extremely effective against *P. berghei*. To be an effective inhibitor of mitochondrial protein synthesis, this drug would have to traverse at least two lipid membranes, the cellular membrane and the mitochondrial membrane. This transport might be facilitated by increased lipid solubility. Indeed, 7-chloro-7-deoxylincomycin was found to have a higher lipid solubility than the parent compound (J. E. Grady, *personal communication*). By introducing certain chemical modifications into antibiotic inhibitors of the ribosome, so as to make them more lipid-soluble as well as preferentially soluble in certain classes of lipids, it may be possible to create new classes of cytotoxic drugs with specificities directed toward a narrow spectrum of cell types.

ADDENDUM IN PROOF

Subunit Specificity of Antibiotic Action

The following table summarizes the sites of action of various antibiotics. In some instances the assignment is tentative or based on negative data. For detailed discussion, refer to the text.

Inhibitors of 30S subunit function	Inhibitors of 50S subunit function	Inhibitor (s) of function (s) requiring both subunits
Tetracyclines Aminoglycosides Bottromycin Pactamycin	Macrolides Lincosaminides Chloramphenicol group Amino acylamino-nucleosides Streptogramin A group Streptogramin B group Sparsomycin	Fusidic acid

Recent Studies on Inhibitors of the 30S Subunit and Effects of Protein Synthesis Inhibitors on RNA Synthesis

Tetracycline. Connamacher and Mandel (25*a*) have continued their experiments on the binding of labeled tetracycline to ribosomes of *E. coli* and *B. cereus*, and they find that binding studies in intact cells and to isolated ribosomes support the conclusion that the drug has a specific attachment to the 30S ribosome subunit.

Aminoglycosides. A new genetic marker for coresistance to neomycin and kanamycin (*nek*) has been reported (8*a*, 8*b*). The available evidence favors a ribosomal site for this mutation, although experiments with sensitive and resistant strains did not show the marked difference in properties that one associates with streptomycin sensitivity and resistance, or spectinomycin sensitivity and resistance, when studied in an in vitro protein synthesizing system. The *nek* mutants were obtained by nitrosoguanidine mutagenesis followed by selection on either kanamycin or neomycin; neomycin is a complex mixture which contains at least three related antibiotics which do not always show cross-resistance (181). The *nek* mutants also showed increased resistance to streptomycin and spectinomycin, two antibiotics which do not normally show cross-resistance. Although mapping by conjugation and transduction suggests that the *nek* locus is near the *spc* locus in the order *nek*, *spc*, *str*, experiments to determine the properties of 30S subunits from *nek* strains have not yet been reported, so it is perhaps premature to classify the *nek* mutation as affecting a 30S ribosomal component.

Recent experiments (126*a*) on genetic mapping of the 30S loci, str^a, spc^a, and K-12 band have established the order of genes as *spc*, *str*, K-12 band, since *str* and K-12 band are cotransducible, but *spc* and K-12 band are not.

Effects of Inhibitors of Protein Synthesis of RNA Synthesis

An extensive study of the effects of a number of inhibitors of protein synthesis (chloramphenicol, tetracycline, streptomycin, spectinomycin, etc.) on the stimulation of RNA synthesis in amino-acid starved bacteria has been carried out (50*a*). We propose that these inhibitors stimulate RNA synthesis by "sparing" amino acids, which depresses RNA synthesis by an unknown mechanism.

Recent Studies on Translocation and Release Factors

Erbe and Leder (48) have studied the products synthesized in a system consisting of salt-washed ribosomes, T-factor, G-factor, initiation factors, and AUG poly U as mRNA. The binding of phenylalanyl tRNA as well as the formation of formylmethionyl phenylalanine (dipeptide) was found to be dependent on added T-factor and initiation factors but not on G-factor. G-factor and GTP, however, were required for polymerization of an additional phenylalanine residue to form tripeptide. This approach provides a possible assay for the translocation reaction.

Pestka (152*a*) has studied the effects of several antibiotics (fusidic acid, chloramphenicol, sparsomycin, and vernamycin A) on the formation of diphenylalanyl tRNA, a process which occurs on ribosomes in the presence of poly U but the absence of supernatant proteins. By supplementing such a system with G-factor and GTP, elongation of the peptide can be obtained with the formation of tri- and higher oligophenylalanines. In these studies it is assumed that elongation beyond the dipeptide is a process which requires "translocation," whereas formation of the dipeptide does not. It was observed that fusidic acid and chloramphenicol exerted no inhibitory effect on diphenylalanine synthesis (and even appeared to stimulate the reaction), whereas sparsomycin and vernamycin A had an inhibitory effect. On the other hand, all four antibiotics inhibited the formation of more highly polymerized product. Since triphenylalanine synthesis in these systems requires GTP and G-factor, it is concluded that fusidic acid specifically inhibits the translocation process. It remains to be seen how specific this inhibition is. There appears to be no significant difference between the inhibitory effects of fusidic acid and of chloramphenicol, as observed in these experiments. Since chloramphenicol does not inhibit the hydrolytic function of the G-factor (26), inhibition of this function is not a necessary condition for inhibition of translocation, as defined by the experiments of Pestka (152*a*).

As pointed out by Pestka (152*a*) two mechanisms of inhibition have been proposed for chloramphenicol. The first is based on inhibition of the puromycin-dependent release (31), and postulates that the inhibitory effect of this antibiotic is due to interference with the actual peptide bond-forming step. The second is based on lack of inhibition of oligopeptide synthesis

[cf. Julian (89), discussed above] and postulates interference with some other step in protein synthesis. Although the present studies represent a refinement over Julian's experimental design (89), the inconsistency still remains. Finally, "translocation" has also been defined operationally in terms of the reactivity of the nascent peptide chain to puromycin in the presence of various antibiotics, as discussed above in connection with the experiments of Cundliffe and McQuillen (31). It was concluded that erythromycin (but not chloramphenicol) was an inhibitor of translocation. These studies are clearly related to the first type of mechanism discussed above.

Scolnick et al. (170*a*) have devised an ingenious assay for chain termination. The assay involves binding of formyl methionyl tRNA to 70*S* ribosomes in the presence of AUG triplet and subsequent release of free formyl methionine on addition of terminator triplet (UAA, UAG, or UGA) and the appropriate protein "release factor" (17). The release reaction was found to be inhibited by tetracycline, streptomycin, sparsomycin, and chloramphenicol, but not by fusidic acid. In terms of the effects of these antibiotics, it was inferred that the termination reaction requires both 30*S* and 50*S* ribosomal subunits since antibiotics which affect each of the subunits are capable of inhibiting this reaction. Moreover, G-factor-dependent GTP hydrolysis, and hence the translocation reaction, does not enter into the release reaction since fusidic acid exerted no inhibitory effect.

The inhibitory effect of streptomycin was interpreted in terms of possible errors induced in the reading of terminator codons. At present, it is not possible to propose an unambiguous unified picture, which accounts for the inhibitory effects of all the antibiotics which were tested in this system. Owing to the nature of the interaction between a triplet (in place of a polymeric messenger) and the ribosome, this system may be over-sensitive and may react to some of the inhibitory effects of the antibiotics tested in a relatively nonspecific way.

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